CULTURE OF ISOLATED EMBRYOS

OF *PINUS RADIATA* D. DON

XUEQIN LIN

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XUEQIN LIN

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Abbreviations

ABA	Abscisic acid
PEG	Polyethylene glycol
GA_3	Gibberellic acid
BA	6-benzyl adenine
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
NAA	α-naphthaleneacetic acid
LP	Modified Quoirin and Le Poivre medium (von Arnold and Eriksson 1981)
SH	Schenk and Hildebrandt (1972) medium
LPSH1	Medium consisting of LP salts and SH vitamins, supplemented with 3% (w/v) sucrose, 5.13 mM L-glutamine, 250 mg/L casein enzymatic hydrolysate and 0.29 μM GA_3, and solidified with 0.8% (w/v) agar.
LPSH2	Medium consisting of half strength of LP salts and SH vitamins, supplemented with sucrose (3%, w/v), casein enzymatic hydrolysate (250 mg/L) and GA_3 (0.58 μM), and solidified with 0.8% (w/v) agar.
BSA	Bovine serum albumin, Fraction V
FW	Fresh weight
SDS-PAGE	Sodiun dodecyl sulfate-polyacrylamide gel electrophoresis
Bis	NN'-methylenebisacrylamide
TEMED	N,N,N',N'-tetra methylethylenediamine
ANOVA	Analysis of variance
GLM	General linear models procedure
SAS	Statistical analysis system
dH_2O	Distilled water

ABSTRACT

Effects of nutritional factors, plant growth regulators, and physical factors on the conversion of isolated embryos into plantlets of *Pinus radiata* were investigated. Results showed that nutritional factors were critical to the conversion of isolated embryos into plantlets of *P. radiata*. The optimum medium strength was half strength of the medium consisting of modified Quoirin and Le Poivre (LP) salts (von Arnold and Eriksson 1981) and Schenk and Hildebrandt (1972) (SH) vitamins. Sucrose (3%) as well as glucose (2-3%) and fructose (2-5%) could serve as carbon sources for the conversion of isolated embryos into plantlets of *P. radiata*. In general, a few significant benefits were found with the addition of organic nitrogen sources tested on the performance of isolated zygotic embryos into plantlets of *P. radiata*.

Nearly all plant growth regulators tested were not beneficial for the conversion of isolated zygotic embryos into plantlets of *P. radiata*, and some of them had negative effect. Only GA_3 (gibberellic acid at 0.58 μM) seemed to stimulate embryos to germinate a little bit earlier in comparison with the control.

Submerging the cotyledons of the isolated embryo into the agar-gelled medium showed better growth in comparison with the control. Embryos cultured in liquid medium grew better but the germination percentage was apparently lower compared with 0.8% agar-gelled medium. Liquid medium with sponge support could increase the percentage of germinated isolated embryos but the embryo growth was not comparable to the liquid medium only. The addition of PEG (polyethylene glycol) 6000 to the liquid medium seemed to increase the germination percentage and had no negative effect on the growth of isolated embryo. Light could influence embryo growth in different ways. For root growth, 16-hour photoperiod appeared to be the best, but for cotyledon development continuous light condition seemed to be the best.

In continuous darkness, the hypocotyl appeared to elongate more, but the cotyledon and root did not grow well.

Isolated embryos cultured on the optimum medium (LPSH2) grew well. The resulting plantlets (i.e. emblings) appeared normal, but were smaller than seedlings. Studies on biochemical changes during germination and early embling or seedling growth showed that the patterns of changes in total protein, soluble sugar, and starch content were generally different between emblings and seedlings. However, on fresh weight basis, total protein concentrations and their SDS-PAGE profiles showed that there was little difference between emblings and seedlings.

Results of this study should be helpful as a basic reference for the artificial seed technology development starting from germination and plant conversion of *P. radiata* somatic embryo with an artificial megagametophyte.

CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Pinus radiata (D. Don) is an exotic conifer species that was originally introduced from California to New Zealand in the mid nineteenth century. Now, 91 percent of New Zealand's plantation forest is *P. radiata* (Cook 2000). Because of the value of forestry to the country, it is the target of a considerable research effort to improve its quality, and hence its value. Biotechnology represents one approach to achieve this aim, and one of its applications can be seen in the supply of raw material, e.g. via micropropagation or somatic embryogenesis (Maddox and Gutierrez 1996).

Somatic embryogenesis is a nonsexual propagation process where somatic (vegetative) cells differentiate into somatic embryos. The technique of multiplying conifers via somatic embryos offers many advantages, both in the mass propagation of selected genotypes and as a part of breeding programs. One of the major advantages of a somatic embryo is that it produces both root and shoot apices during embryogenesis, eliminating the need for a "conventional" tissue culture rooting step (Tautorus *et al.* 1991, Maddox and Gutierrez 1996).

The conversion of somatic embryos or isolated zygotic embryos of *P. radiata* into plantlets /seedlings takes place in the absence of the megagametophyte. This is a major variance from the germination and development of seedlings from the natural seeds. So far, plantlets have been regenerated via somatic embryos in several conifers.

However, the fraction of embryos that germinate vigorously enough to establish autotrophic plants in soil is often unacceptably low (0-70%) (Timmis 1998).

Investigations of the possible contribution of megagametophyte for seedling development in terms of nutritional, hormonal and physical factors are desirable. For this purpose, culture of isolated zygotic embryos of *P. radiata,* which can be obtained more readily and cheaply than somatic embryos, as well as studies on some biochemical changes associated with the conversion of isolated zygotic embryos into plantlets, will be certainly helpful. Ultimately, studies are required to ensure the conversion efficiency of somatic embryos is equivalent, if not better, to that of natural seeds.

1.2 *Pinus radiata*

Pinus radiata (D. Don) was first named in 1837 and has a number of common names including radiata pine and remarkable pine (Bamber and Burley 1983). *P. radiata* is also referred to as Monterey pine, taking this name from Monterey in the coastal region of central California. This is where the major natural occurrence of this species is found (Grut 1970). *P. radiata* also occurs naturally in small numbers on the Guadalupe and Cedros islands off the west coast of Mexico (Perry 1991). *P. radiata* is not highly regarded as a timber species in its place of origin, because of the small quantities of timber available and because the wood is of a poor quality compared with other commercially available species (Bamber and Burley 1983).

P. radiata was first introduced into New Zealand, Australia and Chile in the mid nineteenth century. The species displayed a fast growth rate in a wide range of conditions and showed suitability for a variety of uses (White 1990), and it is now the major plantation forest tree in all three countries, where it is grown for timber and the production of pulp and paper.

The New Zealand environment promotes rapid growth, and with advanced forest management practice including tree breeding, *P. radiata* can produce 650 m^3 of wood or more per hectare, within 25-30 years (Walter *et al.* 1998). Commercial forests currently cover 1.7 million hectares in New Zealand. Of this planted production forest estate, 91 percent is *P. radiata.* The volume of wood available for export is expected to increase dramatically, with about a 74 percent increase between 1996 and 2010. This projected increase assumes 60,000 hectares of new plantings are undertaken each year (Cook 2000).

1.3 Zygotic Embryogenesis *versus* Somatic Embryogenesis

Embryogenesis is the term used to describe the process of embryo formation from the zygote. Typically, conifer embryogenesis comprises distinctive stages, commencing with the formation of a binucleate and then quadrinucleate proembryo immediately after fertilization. Through a series of regular divisions a multicellular embryonal mass is formed, carried above primary and secondary suspensors, and buried within the enveloping female gametophyte. A club-shaped embryo stage follows, and this stage is pushed deep into the gametophyte's corrosion cavity by the coiled suspensor. Shoot and root meristematic regions differentiate on the now torpedo-shaped embryo. During the final stages in the process, cotyledons, hypocotyl, and vascular tissue differentiate and storage product is laid down.

Embryogenesis that does not start with the zygote is called asexual or somatic embryogenesis. That is, besides development from a zygote, embryos can be initiated in cells that are not products of gametic fusion. These embryos are called asexual or somatic embryos and originate in somatic cells or unfertilized gametic cells (Tisserat *et al.* 1979). The earliest report on controlled somatic embryogenesis *in vitro* was in 1958 with carrot (*Daucus carota* L.) tissue cultures (Steward *et al.* 1958). Somatic embryogenesis in conifers was first described with Norway spruce (*Picea abies*) (Hakman and von Arnold 1985, Hakman *et al.* 1985, Chalupa 1985). About the same time Nagmani and Bonga (1985) reported the induction of haploid embryos from the megagametophytic tissue of European larch (*Larix decidua*). Now somatic embryogenesis has been induced in a variety of cycads and conifers (Jain *et al.* 1995). The research on somatic embryogenesis in *P. radiata* was initiated by the New Zealand Forest Research Institute in 1984-85 (Smith *et al.* 1985), and was later being vigorously pursued by Carter, Holt Harvey Ltd. In 1994, a patent had been granted for protocols on somatic embryogenesis and plant regeneration in *P. radiata* (Smith 1994).

Some of the advantages of somatic embryogenesis are: the somatic embryos can be propagated on a large scale in bioreactors, a high yield of plants can be obtained in a short time, the embryos already have a tap root, the embryos can be encapsulated and treated like seeds, true rejuvenation can be obtained even if the somatic embryos are regenerated from mature trees, and the somatic embryos can be cryopreserved. By using somatic embryos in breeding programs, it is possible to keep simultaneously all genotypes cryopreserved until valuable clones have been identified in field tests. The cryopreserved material can then be mass propagated (Egertsdotter 1999).

As the term implies, the process of somatic embryogenesis is similar to zygotic (from the zygote) embryogenesis. Indeed, the published examples of conifer somatic embryogenesis have shown considerable fidelity to the zygotic process. The development of somatic embryos is similar in different coniferous species, and in principle, it resembles their zygotic counterparts during development. It is generally believed that somatic and zygotic embryos show similar morphological and anatomical patterns of development (von Arnold and Hakman 1988*a*, Goldberg *et al.* 1989, Tautorus *et al.* 1991, de Jong *et al.* 1993, West and Harada 1993, Dodeman *et al.* 1997). In addition, several reports have been published on molecular and biochemical changes during the development of somatic and /or zygotic embryos (Goldberg *et al.* 1989, Thomas 1993, Feirer 1995, Kawahara and Komamine 1995, Minocha *et al.* 1999). Furthermore, during germination and early growth the development of somatic embryos also closely resembles that of zygotic embryos (Libby 1986).

1.4 Embryo Culture and Embryo-to-Plant Conversion

Embryo culture is an *in vitro* technique by which plants could be obtained from embryos (including isolated immature /mature zygotic /somatic embryos). Embryo-to-plant conversion is the process of thrifty autotrophic plant formation from the embryo, and is characterized by activation of the root and apical meristems such that root and shoot growth occur (von Arnold and Hakman 1988*b*, Sanoylov *et al.* 1998). In general, embryo-to-plant conversion includes the following events: germination (radicle elongation), development of a vigorous root system, growth and development of the shoot meristem, production of true leaves, a direct shoot-to-root connection, absence of hypertrophy of the hypocotyl, minimization of callus growth in the hypocotyl, and the development of a green plant with a normal phenotype (Redenbaugh *et al.* 1986).

The induction of germination of immature zygotic embryos *in vitro* has been recognised as a useful technique for studies of plant physiology (Raghavan 1986, Bridgen 1994). *In vitro* immature zygotic embryo culture techniques are also a prerequisite for rescuing hybrid embryos resulting from interspecific or intergeneric crosses and showing endosperm failure. For example, in soybean, in order to achieve wide hybridizations between the cultivated soybean [*Glycine max* (L.) Merr., subgenus Soja] and its wild relatives belonging to the subgenus *Glycine* many efforts were made to develop protocols for the culture of immature zygotic embryos ensuring high plant recovery (Vagera and Hanačkova 1979, Newell and Hymowitz 1982, Tilton and Russell 1984, Singh and Hymowitz 1985*a, b*, Singh and Hymowitz 1987). Lippmann and Lippmann (1993) investigated the factors influencing *in vitro* growth rates of soybean embryos using embryos isolated at the cotyledon stage, and proposed a protocol for culturing soybean cotyledon stage embryos under conditions ensuring high plant recovery. Interest in experimental embryology in cereals has led to the development of protocols for the isolation and culture of zygotes from *Zea mays* (Leduc *et al.* 1996), *Hordeum vulgare* (Holm *et al.* 1994) and *Triticum aestivum* (Holm *et al.* 1994, Kumlehn *et al.* 1997, 1998). Results from the experiments of

Iglesias *et al.* (1994) showed that wheat proembryos excised 7 days after anthesis could germinate to normal fertile plants with an efficiency of 90%. Kumlehn *et al.* (1998) reported the regeneration of wheat zygotes through direct embryo differentiation, and 90% of the zygote developed into plants. Recently, Zhang *et al.* (1999) presented a protocol for efficient plant regeneration from the isolated zygotes of rice (*Oryza sativa* L.), which provided a basis for an experimental approach to zygote transformation and could be applied in marker gene-free transformation.

In conifer, few investigations have been done on immature zygotic embryo culture. Brown and Gifford (1958) studied the relation of the cotyledons to root development of excised mature zygotic embryos of *Pinus lambertiana* Dougl grown *in vitro* and found that nutrient supply to the cotyledons was important, affecting both rate and duration of root growth. Experiments on mature zygotic embryos excised from *Pinus radiata* seeds showed that embryos developed with normal morphology, although not as large as those from natural seeds, when cultured in a small volume of a nutrient medium within a small aluminium capsule (Teasdale and Buxton 1986).

Although plantlets have been regenerated via somatic embryos in several coniferous species (Hakman and von Arnold 1985, Chalupa 1985, Gupta and Pullman 1993, Smith 1994), the yield of fast-growing plantlets has so far been rather poor and remains a limitation to the implementation of this technology (Dunstan 1988, Tautorus *et al.* 1991, Timmis 1998). A common problem for low germination rates of mature somatic embryos is that the mature somatic embryos can only form cotyledons and a hypocotyl, but poor or no root development (Egertsdotter 1999). Many researchers have aimed to improve the maturation and germination responses of conifer somatic embryos for mass propagation. Earlier on only about 1-2% of the somatic embryos was reported to develop into plantlets. After employing some treatments the efficiency of somatic embryo yield and plantlet production from somatic embryos was improved. For example, it has been shown that embryo production from spruce cultures and subsequent embryo maturation was enhanced by incorporating abscisic acid into the medium (Becwar *et al.* 1987, von Arnold and

Hakman 1988*b*, Dunstan *et al.* 1988, Roberts *et al.* 1990, Webster *et al* 1990). The abscisic acid treatment on mature black spruce somatic embryos was superior with respect to desiccation tolerance and to the quality of germinants (Beardmore and Charest 1995). Conversion of somatic embryos was considerably improved (from 14 to 65%) by mild desiccation treatment at 97% relative humidity (Roberts *et al.*1991). Webster *et al* (1990) investigated seventy-one lines (genotypes) of embryogenic cultures from six open-pollinated families obtained by culturing immature embryos of interior spruce. They demonstrated that treatment of mature embryos with a high relative humidity resulted in partial drying of the embryos and upon rehydration, germination of the eight genotypes tested was markedly enhanced. Within one week of being placed under germination condition, somatic embryos treated with the high relative humidity treatment showed 80-100% germination for twelve of the genotypes, and most genotypes had germination rates of greater than 40%. However, Bomal and Tremblay (1999) reported that for all five genotypes of black spruce tested, desiccated embryos (applying optimal desiccation protocol using large desiccation chamber at 97% relative humidity and a constant embryo mass of 40 mg embryos plus 40 mg water) gave plantlet regeneration rates similar to the control undesiccated embryos. Timmis (1998) thought that to apply somatic embryogenesis to clonal propagation of conifers, the main current challenges yet to overcome are the low frequencies of embryo maturation and their conversion into viable plantlets, as well as the high costs associated with converting cloned embryos to plants.

In fact, the performances of somatic embryos are similar to isolated zygotic embryos. For example, Teasdale and Buxton (1986) demonstrated that the performances of isolated zygotic embryos were impaired when cultured in liquid or agar medium. David *et al.* (1995) found similar behaviours between somatic embryos and zygotic embryos in *Pinus caribaea*, that is both of their seedlings were smaller than seedlings derived from mature seeds. Attree *et al.* (1994) also reported that the length of germinant was closely related with embryo type. They observed that the length of plantlets germinated from excised zygotic embryos or somatic embryos was not as great as for plantlets recovered from whole seeds. Therefore, investigations of the possible contribution of megagametophyte for seedling development by using the culture of isolated zygotic embryos of *P. radiata* and finally to manipulate them by providing artificial megagametophyte (i.e. optimum nutritional components and their carriers) should be of interest to the aim of large-scale multiplying *P. radiata* via somatic embryos.

1.5 Biochemical Changes during Embryo-to-Plant Conversion

Conversion means the establishment of germinated somatic embryos into soil and the capacity for autotrophic growth. Germination of fully developed and mature somatic embryos is the vigorous growth and development into plants ready to make the transition to autotrophic growth, and therefore, is the first phase of conversion. There are several reports on somatic embryogenesis and plantlet regeneration in conifers. However, there are very few reports on somatic seedlings (plantlets derived from somatic embryos) established in soil (Gupta and Grob 1995).

Seed germination begins with imbibition and ends with the start of elongation, usually of the radicle. Events during germination include protein hydration, subcellular structural changes, increased respiration, macromolecular synthesis and cell elongation (Bewley and Black 1985); seedling growth begins when germination finishes. Desiccation, an essential part of normal seed development, may activate genes for germination and turn off the synthesis of embryogenesis-related proteins (Misra and Bewley 1985, Kermode 1990). Additional mRNAs are stored during development for immediate availability upon rehydration, for translation of the proteins required for germination (Delseny *et al.* 1977, Harada *et al.* 1988).

In conifers, the megagametophyte is the main tissue containing storage reserves mainly in the form of lipids and proteins. These hydrolysed reserves are transported to the embryo from the megagametophyte where they are used to support the growth of the embryo (de Carli *et al.* 1987). The biochemical changes that occur in germinating conifer seeds have been investigated at a number of levels (Misra 1994), for example, in *Pinus contorta* (Dougl.) zygotic embryos (Gifford *et al.* 1991). However, few similar germinating events have been described in conifer somatic embryos. During plantlet regeneration, the storage proteins and lipids of the somatic embryos were rapidly mobilized in a manner similar to that in zygotic embryos and germination-related gene activity was observed (Attree *et al.* 1992, Misra *et al.* 1993). Treatments that promote storage-reserve accumulation in somatic embryos of conifers may contribute to their desiccation tolerance. So far, studies of mRNAs during desiccation and germination of somatic embryos of conifers have not been reported. In zygotic embryos of angiosperms, desiccation treatment is required to turn off storage protein gene expression (Finkelstein *et al.* 1985, Finkelstein and Crouch 1986) and to activate genes for germination. In the absence of such a treatment, the developmental and germination events appear to overlap. Such a situation in non-quiescent somatic embryos may be the cause of poor post-germinative vigour, whereas partial or complete drying of somatic embryos, which leads to synchronization of germination, may be effective in turning off the development-associated gene activity. Partially or fully dried somatic embryos show good synchronization of root and shoot elongation (Attree *et al.* 1992). Poor post-germinative vigour of somatic embryos may be due in part to a failure to completely break dormancy (Gray and Purohit 1991). Seeds of many conifers commonly require stratification for several weeks or months at 1-5°C to be released from dormancy. Stratification increased the translational activity of stored mRNA in the earliest stages of germination of *Pinus strobus* (L.) seeds (Whitmore 1991). Cold stratification may also reduce endogenous abscisic acid levels, thereby allowing plant development to proceed. Drying during maturation may also reduce endogenous levels of abscisic acid within embryos and seeds of some plants (Kermode 1990).

Post-germinative growth of conifer somatic embryos or isolated zygotic embryos occurs without the benefit of the megagametophyte, which is a major organ for storage of both lipids and proteins within the conifer seed. Conifer somatic embryos therefore require nutrients, usually in the form of plant growth regulator free media containing 2-3% sucrose (von Arnold and Hakman 1988, Becwar *et al.* 1989), but the *in vitro* germination medium may not entirely supplant the role of the megagametophyte during germination and early growth (Cyr *et al.* 1991). Gupta *et al.* (1993) described the efficient conversion of conifer somatic embryos to seedling by planting them directly into sterile soil mix wetted with sucrose containing medium. Following germination and early growth of somatic embryos, both storage triacylglycerol (Attree *et al.* 1992) and storage polypeptides (Cry *et al.* 1991, Misra *et al.* 1993) were rapidly used in a manner similar to that in zygotic embryos. With respect to *in vitro* systems, then, the composition of the germination medium takes on special importance, as it must be a substitute for the megagametophyte to supply adequate amounts of nitrogen and carbon skeletons. It must be remembered that even a "high quality somatic embryo", having abundant protein and triglyceride reserves, depends in large part upon the nutrients supplied by the medium, just as the zygotic embryo is dependent upon the megagametophyte. Studies on some biochemical changes associated with the conversion of isolated embryos into plantlets of *P. radiata*, as presented in this thesis, will be useful in designing an optimised germination medium for *P. radiata* somatic embryos.

1.6 Synthetic Seed Production

Synthetic or artificial seeds are functionally defined as somatic embryos engineered to be of use in commercial plant production (Gray and Purohit 1991). Synthetic seed technology is designed to combine the advantages of clonal propagation with those of seed propagation. The potential uses for artificial seeds are numerous, including delivery of elite germplasm, hand-pollinated hybrids with reduced seed fertility, and genetically engineered plants with sterile or unstable genotypes. The size of the synthetic seed and the coating around the somatic embryo are advantageous potential for storage, handling, transportation and planting (Redenbaugh *et al.* 1988).

Synthetic seed consists of either a quiescent or nonquiescent somatic embryo with or without a protective encapsulation (Gray and Purohit 1991). There are several types of synthetic seeds, but the ideal synthetic seed maybe a dry somatic embryo encapsulated with a artificial endosperm /megagametophyte, containing nutrient reserves, antibiotics, fungicides and plant growth regulators to promote early growth and survival. This unit could also be encapsulated further with a synthetic seed coat to enable mechanical handling and planting (McKersie and Bowley 1993).

During the mid-1970's, one group at Union Carbide led by Robert Lawrence began to consider various methods for cloning forest trees. This group focused on delivery of somatic embryos using fluid drilling technology (Lawrence 1981). However, fluid drilling of somatic embryos do not have the advantages that true seeds possess such as long-term storage, ease of transportation, and planting with existing equipment. Therefore, a true artificial seed may be more attractive for large-scale planting of woody plantation species where large numbers are usually sown in a short time. The encapsulation of somatic embryos was first reported by Redenbaugh *et al.* (1984). Later many significant research studies have been done on artificial seed technology, but so far there have been no somatic embryo derived plants deployed in commercial quantities using this technology for any plant species. The requirements of this system are complicated and are still undergoing intensive research and development. Most systems use alginate gels or other types of artificial seed coating systems (Redenbaugh 1986*a, b*, Sakamoto *et al.* 1991, Fujji *et al.* 1992, Lulsdorf *et al.* 1993, Ara *et al.* 1999), which work fairly well but only on an experimental scale.

In some woody species such as conifers, the normal zygotic seed contains the embryo along with nutritive tissues of the megagametophyte. Because these species do not have storage reserves in the cotyledons, they cannot germinate autotropically and require the support of the megagametophyte during the germination process. Thus any artificial seed for these species will require that an artificial megagametophyte be present too. There has been some progress in the development of a nutrient source within an encapsulation matrix (Sakamoto *et al.* 1991, Mala. *et al.* 1995, Nieves *et al.* 1998), but additional research is needed to make this commercially feasible, especially in woody species. The embryo must be oriented correctly within an artificial megagametophyte and the embryo /megagametophyte complex must be packaged in a matrix material that will allow water uptake without drying out. This entire package must eventually break open and be shed similar to the natural seed coat (Sakamoto *et al.* 1992). An important component of this type of artificial seed is artificial megagametophyte. Therefore, investigations of the possible contribution of megagametophyte for seedling development in terms of nutritional, hormonal and physical factors, by using the culture of isolated zygotic embryos of *P. radiata,* as presented in this thesis, will provide a reference for constructing somatic embryo radiata pine with an artificial megagametophyte.

1.7 Aims and Objectives

The overall aim of this thesis research was to provide some insights into the *in vitro* requirements for the efficient conversion of somatic embryos into plantlets of *P. radiata*. More specifically, the objectives of this project were to:

1. investigate factors that are important for the conversion of isolated zygotic embryos into plantlets of *Pinus radiata.*
2. determine the optimum requirements for isolated zygotic embryos of *Pinus radiata* during their growth into plantlets.
3. study some biochemical changes associated with the conversion of isolated zygotic embryos into plantlets of *Pinus radiata.*

CHAPTER TWO

MATERIALS AND METHODS

2.1 Source of *Pinus radiata* Seeds

Seeds of *P. radiata* tested in this study were obtained from New Zealand Tree Seeds (Rangiora, New Zealand) and stored at 4°C in a sealed plastic bag.

2.2 Source of Chemicals

The following chemicals were purchased from British Drug Houses Ltd. (B.D.H.), England: Tween 20, arginine monohydrochloride, glucose, fructose, silver nitrate ($AgNO_3$), polyethylene glycol (PEG) 6,000, acrylamide, NN'-methylenebisacrylamide (bis), phenol, glycerol, iodine, potassium iodide, sodium dodecyl sulfate (SDS), perchloric acid, bovine serum albumin, Fraction V (BSA), and 2-mercaptoethanol.

Some chemicals were bought from Sigma (USA), including maltose, L-glutamine, casein enzymatic hydrolysate, gibberellic acid (GA_3), putrescine, spermidine, 6-benzyl adenine (BA), kinetin, indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), soluble potato starch, and Trizma Base.

Ammonium persulphate, N,N,N',N'-tetra methylethylenediamine (TEMED), and molecular weight standards were bought from Bio-Rad, California, USA.

Some media were solidified with agar bought from Germantown, New Zealand.

2.3 Factors Influencing the Conversion of Isolated Embryos of *P. radiata*

2.3.1 Seed Sterilisation and Stratification

P. radiata seeds were surface sterilised by soaking them in a diluted bleach solution containing 1% (v/v) sodium hypochlorite and Tween 20 (at 2 drops per 100 mL) for 15 minutes, rinsed in running water for 24 hours, and placed in a sealed plastic bag and refrigerated for stratification (i.e. storage at 4°C for 2 weeks). After the low-temperature treatment, under aseptic conditions, seeds were surface sterilised again by soaking them in the same solution for 10 minutes, and rinsed three times with sterile distilled water. Seed coats were removed, the naked seeds were surface sterilised further in 5% (v/v) hydrogen peroxide for 10 minutes, and rinsed three times with sterile distilled water.

2.3.2 Culture of Isolated Zygotic Embryos

The embryos were aseptically isolated free of the megagametophyte and other seed structures without apparent injury (Plate 1). They were cultured under a range of different media variations (see 2.3.3).

Unless stated otherwise, embryos were placed horizontally on the surface of media in a growth room under continuous lighting with cool white fluorescent tubes (approximately 150 μmol $m^{-2} \cdot s^{-1}$) at 22 ± 1°C.

If not otherwise mentioned the basal medium named 'LPSH1-medium' consisted of modified Quoirin and Le Poivre (LP) medium salts (von Arnold and Eriksson 1981), and Schenk and Hildebrandt (1972) (SH) medium vitamins, supplemented with 3% (w/v) sucrose, 5.13 mM L-glutamine, 250 mg/L casein enzymatic hydrolysate and

0.29 μM gibberellic acid (GA_3), and was solidified with 0.8% (w/v) agar. The pH of all media was adjusted to 5.6-5.8 (Metrohm Herisau E350 B, Switzerland) with 1 M NaOH or HCl prior to autoclaving for 20 minutes at 121°C, 103.5 kPa.

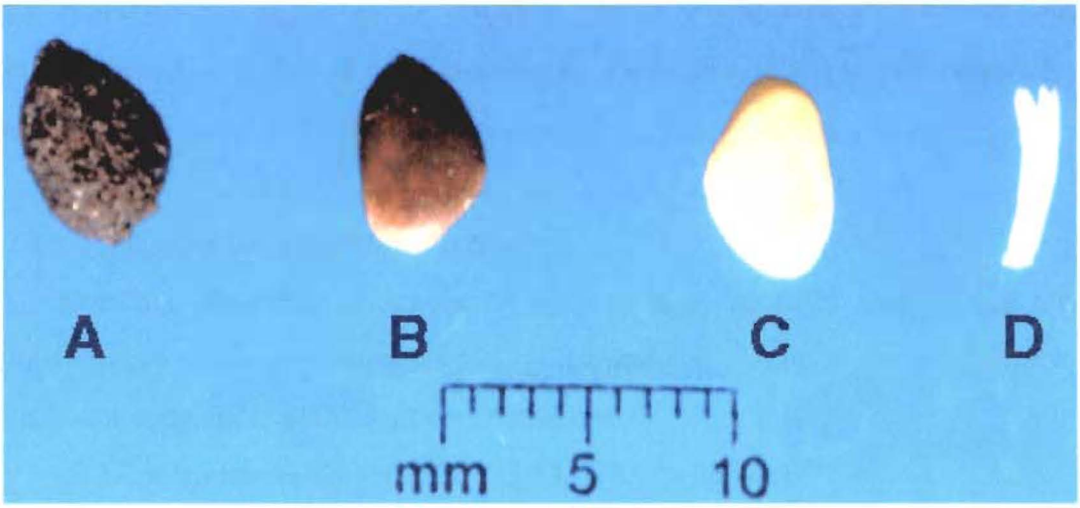


Plate 1. A photograph of intact seed (A), seed without hard seedcoat (B), seed without hard and soft (inner papery membrane) seedcoats (C), and isolated embryo (D) of *Pinus radiata.*

2.3.3 Variations of Media

2.3.3.1 Experimental Design

A completely randomized design with subsampling was employed in this study. Seed sterilisation, stratification, isolation and culture of the embryos, and the basal medium were as described in 2.3.1 and 2.3.2. There were 6 replicates per treatment, each consisting of 3 embryos.

2.3.3.2 Effect of Medium Strength

Five different medium strengths were tested in this study: 0, 0.25, 0.5, 1.0, and 2.0 times those of modified Quoirin and Le Poivre (LP) medium salts (von Arnold and

Eriksson 1981), and Schenk and Hildebrandt (1972) (SH) medium vitamins. The other components in all five media tested were the same as in the basal medium described in 2.3.2.

2.3.3.3 Influence of Carbohydrates

To determine the influence of different carbon sources, the basal medium was supplemented with one of the four carbohydrates (sucrose, glucose, fructose, and maltose) each at six different concentrations (0, 1, 2, 3, 4, and 5%, w/v).

2.3.3.4 Effect of Organic Nitrogen Sources

To investigate the effect of organic nitrogen sources, the basal medium was altered with respect to the concentration of L-glutamine and casein enzymatic hydrolysate. Each was tested at five different concentrations:

L-glutamine (mM): 0.00, 2.57, 5.13, 7.70, 10.27; and

casein enzymatic hydrolysate (mg/L): 0, 125, 250, 375, 500.

To investigate further the effect of organic nitrogen sources, the LPSH1-medium was supplemented with one of the following three organic nitrogen sources each at five different concentrations:

arginine monohydrochloride (mM): 0.00, 1.78, 3.56, 5.34, 7.12;

putrescine (μM): 0.00, 0.16, 0.31, 0.62, 1.24; and

spermidine (μM): 0.00, 0.03, 0.07, 0.14, 0.28.

2.3.3.5 Effect of Plant Growth Regulators

To examine the effect of different plant growth regulators, the basal medium was supplemented with one of the following plant growth regulators each at five different concentrations were used:

GA_3 (gibberellic acid) (μM): 0.00, 0.14, 0.29, 0.58, 1.15;

BA (6-benzyl adenine) (μM): 0.00, 0.06, 0.11, 0.22, 0.4;.

Kinetin (μM): 0.00, 0.12, 0.23, 0.46, 0.93;

IBA (indole-3-butyric acid) (μM): 0.00, 0.06, 0.12, 0.25, 0.49;

IAA (indole-3-acetic acid) (μM): 0.00, 0.07, 0.14, 0.29, 0.57; and

NAA (α-naphthaleneacetic acid) (μM): 0.00, 0.07, 0.13, 0.27, 0.54.

2.3.3.6 Effect of Ethylene

To determine if ethylene could affect the growth of embryos, five different concentrations of $AgNO_3$ (silver nitrate, an ethylene action inhibitor) were added to the LPSH1-medium: 0.00, 2.94, 5.89, 11.77, and 23.55 μM.

2.3.3.7 Comparison of Different Ways to Place the Embryos on Medium

The embryos were planted in the LPSH1-medium in three different ways: (1) embryos were horizontally placed on the surface of the medium, (2) the cotyledons were inserted into the medium leaving the hypocotyl and radicle end free, and (3) with the radicle end and basal part of the hypocotyl submerged in the medium leaving the upper part of the hypocotyl and cotyledons free. For treatment (2) the containers were inverted during the culture period so that radicles were always growing downwards.

2.3.3.8 Comparison of Agar-gelled and Liquid Media

Three different treatments were compared in this study: (1) 0.8% (w/v) agar-gelled medium, (2) liquid medium (half of the volume normally used, ca. 6 mm thick), and (3) polyurethane sponge (ca. 15 mm thick) floated on liquid medium. The basal medium was used in all 3 treatments. For treatment (2) embryos were floated on the surface of the liquid medium at the beginning of culture period. For treatment (3) embryos were placed on the surface of the sponge.

2.3.3.9 Effect of PEG (polyethylene glycol)

To investigate further the possible osmotic effect of liquid medium on the conversion of embryos into seedlings, five different concentrations of PEG 6,000 were added to the basal medium: 0, 1, 3, 5, and 7% (w/v). For this experiment embryos were floated on the surface of the liquid medium at the beginning of culture period.

2.3.3.10 Influence of Light

Three different treatments were compared in this study: (1) continuous light, (2) 16-h daily photoperiod, and (3) continuous darkness.

2.3.4 Data Collection

Root and shoot lengths were scored every 7 days up to day 21. Embryos were scored as germinated if they exhibited both shoot and root elongation. Those that did not germinate by day 21 also appeared nonviable (i.e. they became shrivelled and brown). Shoot growth and root growth were measured if the cotyledons or hypocotyl region elongated by more than 2 mm, and if root elongation was greater than 2 mm, respectively. Germination percentage was calculated as the total number of embryos that germinated divided by the total number of embryos tested in a replicate multiplied by 100. The seedling fresh weight and the weight after drying at 70°C for 24 hours were also determined at the end of the experiment, i.e. day 21.

2.4 Seed Germination Test on Filter Paper

Seeds, which had been sterilised and stratified (see 2.3.1), were placed onto four layers of moist filter paper in Petri dishes (90 mm diameter). Three treatments in this experiment were: (1) intact seeds, (2) seeds without hard seedcoat, (3) seeds without both hard and soft (inner papery membrane) seedcoats (Plate 1). Dishes were sealed with plastic film and incubated under conditions identical to the culture of the isolated zygotic embryos (2.3.2). There were four replicates per treatment each consisting of 30 seeds.

The number of seeds germinated was scored every 7 days up to day 21. The germinated seeds were those that exhibited both shoot and root growth. Shoot growth was measured if the cotyledons or hypocotyl region elongated by more than 2 mm, and root growth was measured if root elongation was greater than 2 mm. Germination percentage was calculated as the total number of seeds that germinated divided by the total number of seeds tested in a replicate multiplied by 100.

2.5 Biochemical Changes Associated with the Conversion of Isolated Embryos of *P. radiata*

On the basis of the experiments in 2.3 and 2.4, an optimum medium consisting of half strength of modified Quoirin and Le Poivre (LP) salts (von Arnold and Eriksson 1981), and Schenk and Hildebrandt (1972) (SH) vitamins solidified with 0.8% (w/v) agar and supplemented with sucrose (3%, w/v), casein enzymatic hydrolysate (250 mg/L) and GA_3 (0.58 μM), named 'LPSH2-medium' was developed for the routine culture of isolated zygotic embryos of *Pinus radiata*. They were cultured in this medium under continuous light at 22 ± 1°C, and harvested for various biochemical analyses (2.5.1 and 2.5.2).

Time course of biochemical changes were also studied in both seeds without hard seedcoat, and isolated zygotic embryos cultured on 0.8% (w/v) water agar medium (i.e. without any nutritional or plant growth regulator supplements) under conditions identical to the above.

2.5.1 Time Course of Total Protein Changes

Whole isolated embryos, emblings (plantlets that were produced from isolated embryos), or seedlings (plantlets that were produced from seeds) were harvested after 0, 7, 14, and 21 days in culture and blotted dry. Except for day 0, the emblings /seedlings were excised into cotyledons, hypocotyls and roots. Their fresh weights and lengths were measured at each harvesting time. For embryos cultured on water-agar medium, whole explants were used at each time. Three replicates each comprising 5-30 embryos or embling /seedling parts (depending upon size of the plant materials at the time of harvest) were collected at each time of harvesting. All plant materials were pooled and stored at –20°C. The frozen plant materials were ground to a fine powder, when required, with liquid nitrogen in a pre-chilled mortar. The powder was extracted with a buffer containing 125 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 2% (w/v) SDS, and 5% (v/v) 2-B-mercaptoethanol, in a ratio of 0.10 g fresh weight of plant material to 0.75 mL extraction buffer. After leaving the extracts on ice for 10 min, they were centrifuged at 15000 rpm, 4°C for 15 minutes (Eppendorf 5403, Germany). The supernatants were collected and stored at –20°C.

2.5.1.1 Protein Concentration Determination

The total protein concentration in the supernatants were determined using Coomassie blue dye G-250 (Bradford 1976). In a typical microassay, 0.1 mL extract was added to 1 mL the dye and left standing at room temperature for 5 minutes. The absorbance of each sample was measured at 595 nm and the protein concentration was read from a standard curve established using bovine serum albumin, Fraction V (BSA in short).

2.5.1.2 SDS-PAGE of Proteins

The protein contents in the supernatants were also analyzed using single-dimensional sodiun dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). It was carried out in 0.75 mm slab gels of 10% (w/v) acrylamide on a vertical electrophoresis unit using the method of Laemmli (1970). To each lane, 10 μg protein was applied. Molecular weight determinations were as outlined in Weber and Osborne (1969) and the mixture of molecular weight markers consisted of phosphorylase B, 97 400; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; and soybean trypsin inhibitor, 21 500; and lysozyme, 14 400 (the numbers were molecular weight in Daltons). Gels were stained with Coomassie blue R-250 and then destained for visualization of protein bands.

2.5.2 Time Course of Soluble Sugar and Starch Changes

Plant materials were collected and stored at –20°C as described 2.5.1. The frozen plant tissues were ground to a fine powder with liquid nitrogen in a pre-chilled mortar. The powder was extracted with 80% (v/v) ethanol and centrifuged at 11000 rpm, 2°C for 15 minutes (Eppendorf 5403, Germany). The resulting pellet was re-extracted 3 times and the supernatants were pooled into a clean glass vial. The total volume of 80% (v/v) ethanol used was in a ratio of 0.10 g fresh weight of plant material to 2 mL 80% (v/v) ethanol. The ethanol was evaporated off from the vial kept in a 60°C water bath in a fume hood until dryness. The residue was re-dissolved in distilled water (dH_2O), 1 mL per 0.10g fresh weight of plant material. This solution was analyzed for soluble sugar content (2.5.2.1).

2.5.2.1 Soluble Sugar Assay

Soluble sugar content was determined using the method of Dubois *et al.* (1956). To 0.5 mL of a solution for soluble sugar assay, 0.5 mL of 5% (w/v) phenol and 1.0 mL of concentrated H_2SO_4 were added and the solution was thoroughly mixed. The absorbance of each sample was measured at 490 nm and soluble sugar content was calculated from a glucose standard curve.

2.5.2.2 Starch Assay

Modification of a method for starch determination described by Bewley *et al.* (1993) was used. Starch was solubilized from the resulting pellet (2.5.2) with 30% (v/v) perchloric acid in a ratio of 0.10 g fresh weight of plant material to 1 mL 30% (v/v) perchloric acid. The slurry was left at room temperature for 24 hours for starch extraction. The amount of amylose was estimated using an iodine reagent (0.1 mL iodine stock with 9.9 mL of 0.05 M HCl freshly prepared). To 0.5 mL of the starch solution, 0.5 mL of iodine reagent and 1.0 mL of 30% (v/v) perchloric acid were added and the solution was vortexed. The absorbance of each sample was measured at 620 nm and its starch content was calculated from a standard curve established using soluble potato starch.

2.6 Data Analysis

Data collected from all the experiments were subjected to analysis of variance (ANOVA) using the GLM procedure of Statistical Analysis System (SAS) version six

(SAS Institute Inc., USA). Percentages were arcsine transformed before analysis. A one-way or two-way ANOVA was preformed on each data set at the 95% or 99% significance level. When the ANOVA indicated statistical significance, Duncan's Multiple Range Test was preformed at the 95% significance level to distinguish between treatments. The standard error of the mean was calculated for each sample using the spreadsheet program Microsoft Excel 97 (Microsoft Corporation, USA).

CHAPTER THREE

RESULTS

3.1 Factors Influencing the Conversion of Isolated Embryos of *P. radiata*

3.1.1 Nutritional Factors

3.1.1.1 Strength of the Medium

Germination percentage of isolated embryos of *P. radiata* was affected significantly by the medium strength ($P<0.01$) (Figure 1). Very few isolated embryos could survive on the medium without salts and vitamins. A slightly higher percentage of isolated embryos germinated on half strength (0.5x) medium than on the 0.25, 1 or 2x medium strengths throughout the study period. Embryo germination was delayed when placed on the double strength (2x) medium which did not gel well.

The maximum lengths of cotyledon, hypocotyl, root and whole seedling were all achieved by embryos cultured on half strength (0.5x) medium. The effect of this medium strength was significantly different from that of other medium strengths (Table 1). The fresh and dry weights of whole seedling were significantly lower in the treatments with medium strength lower than 0.25x, while there were no differences among 0.5x, 1x and 2x medium strengths.

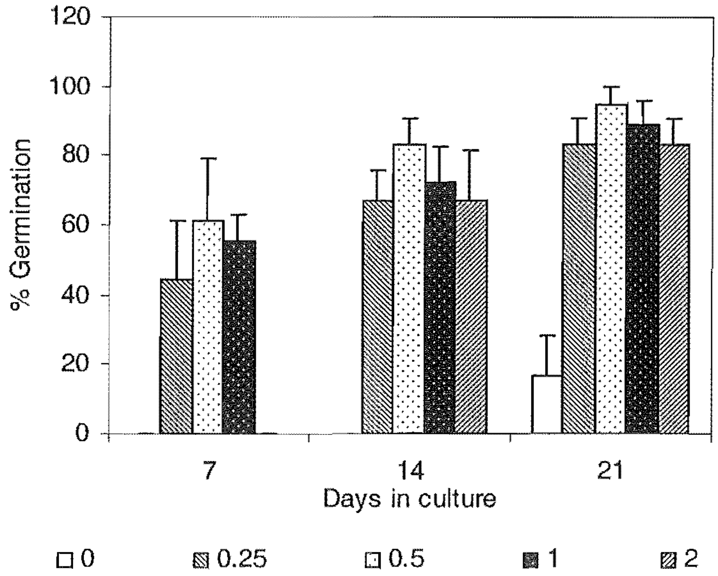


Figure 1. Effect of different medium strengths on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 1. Effect of different medium strengths on the growth of isolated embryos of *Pinus radiata*. All data were obtained at day 21

Medium	Mean length (mm)				Mean weight (mg)	
strength	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0x	3.4 c	5.5 c	1.3 c	10.1 c	40.1 b	5.2 b
0.25x	6.1 b	12.0 ab	10.9 ab	29.0 b	44.1 b	6.2 b
0.5x	9.3 a	13.6 a	17.6 a	40.5 a	76.6 a	8.5 a
1x	7.4 ab	11.7 ab	12.5 ab	31.7 ab	69.9 a	8.3 a
2x	8.9 a	9.7 b	8.3 b	26.9 b	80.2 a	9.4 a

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

3.1.1.2 Carbohydrates

Carbohydrates were very important for the *in vitro* culture of isolated embryos of *P. radiata*. Sucrose as well as glucose or fructose could serve as carbon sources for isolated embryos of radiata pine cultured *in vitro* (Figures 2-4, Tables 2-4). A significant effect of the concentrations of sucrose, glucose or fructose on both germination percentage and growth of isolated embryos of *P. radiata* was found (P<0.01). Isolated embryos could not germinate on the medium without any sugars. Only a few isolated embryos grew on the medium with 1% (w/v) sucrose after 21 days in culture. More embryos germinated at 3% (w/v) sucrose, 2-3% (w/v) glucose or 2-5% (w/v) fructose throughout the study period.

The lengths of cotyledon, hypocotyl, root and whole plantlet as well as the fresh and dry weights were greatest when embryos were cultured on medium supplemented with 3% (w/v) sucrose (Table 2). The best concentrations of glucose for cotyledon, hypocotyl and root growth were 2-3% (w/v) (Table 3). Isolated embryos generally grew well on medium supplemented with 1-4% (w/v) fructose (Table 4). Although maximum fresh and dry weights were recorded when embryos were cultured on medium supplemented with 5% (w/v) fructose, here the plantlets grew a little bit abnormal, e.g. shorter and thicker.

Maltose as a carbon source was not so effective as sucrose, glucose or fructose in supporting the growth of isolated embryos of radiata pine (Table 5). The germination percentage at day 21 was not higher than 39% in most maltose-containing media (Figure 5).

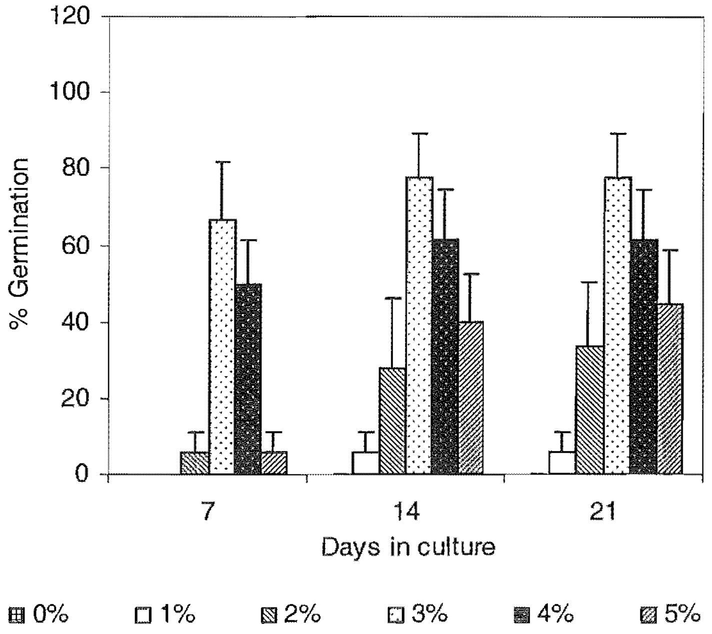


Figure 2. Effect of different sucrose concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 2. Effect of different sucrose concentrations on the growth of isolated embryos of *Pinus radiata*. All data were obtained at day 21.

Sucrose	Mean length (mm)				Mean weight (mg)	
(%)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0	0.0 b	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c
1	0.7 b	0.6 c	0.2 c	1.4 c	2.79 c	0.4 c
2	3.3 ab	6.3 b	4.2b c	13.8 b	35.8 b	4.0 b
3	7.4 a	11.9 a	13.2 a	32.5 a	71.5 a	8.1 a
4	6.6 a	7.8 b	11.2 a	25.6 ab	48.6 b	6.4 ab
5	5.1 a	7.1 b	8.4 ab	20.6 ab	46.8 b	5.9 ab

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

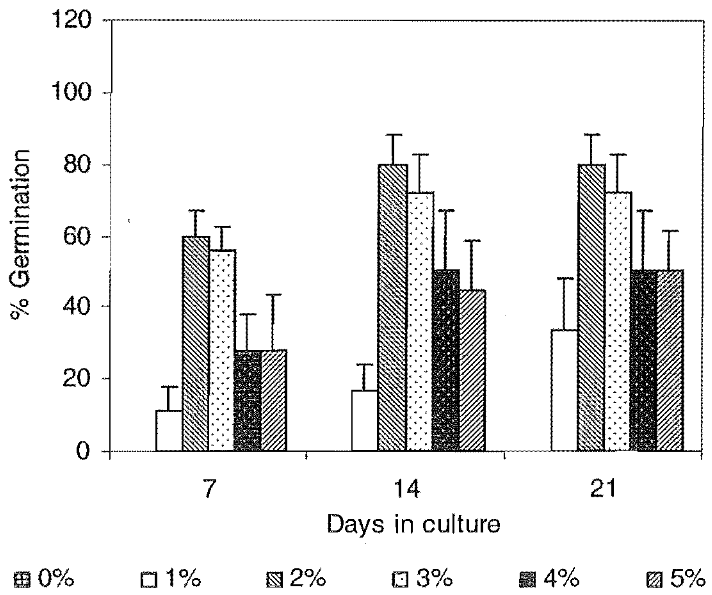


Figure 3. Effect of different glucose concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 3. Effect of different glucose concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

Glucose	Mean length (mm)				Mean weight (mg)	
(%)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0	0.0 c	0.0 d	0.0 c	0.0 c	0.0 c	0.0 c
1	4.9 ab	6.1 bc	3.4 bc	14.4 b	27.4 b	3.1b c
2	8.1 a	10.2 a	10.8 ab	29.1 a	57.7 a	6.7 a
3	6.1 ab	9.2 ab	15.4 a	30.8 a	64.1 a	7.9 a
4	2.9 bc	5.3 c	8.7 abc	16.8 ab	40.4 ab	5.0 ab
5	4.5 b	6.7 bc	10.1 abc	21.2 ab	41.3 ab	6.5 a

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

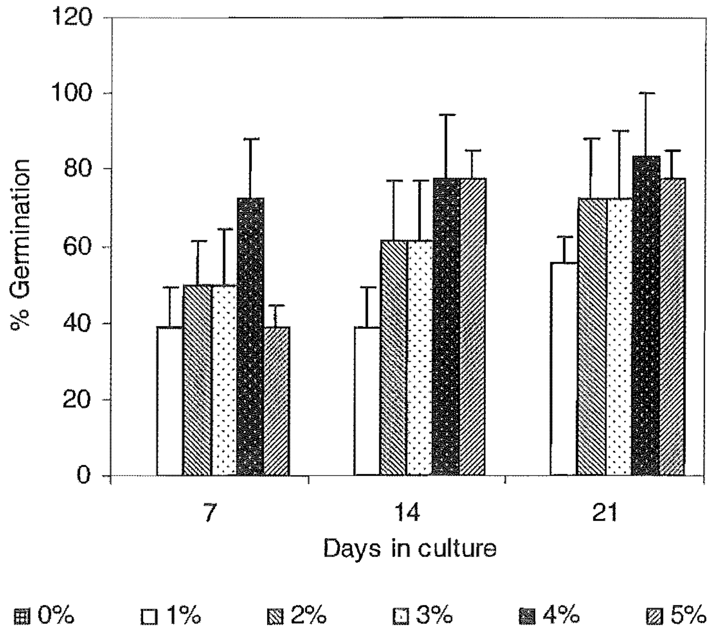


Figure. 4. Effect of different fructose concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 4. Effect of different fructose concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

Fructose	Mean length (mm)				Mean weight (mg)	
(%)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0	0.0 b	0.0 b	0.0 d	0.0 c	0.0 c	0.0 c
1	4.7 a	7.6 a	6.2 dc	18.4 bc	34.7 b	3.6 bc
2	6.3 a	10.2 a	11.2 bc	27.7 ab	54.6 ab	6.7 ab
3	7.5 a	10.6 a	17.6 ab	35.7 ab	62.9 ab	7.3 ab
4	8.4 a	9.9 a	23.0 a	41.4 a	64.0 ab	8.3 a
5	8.6 a	9.3 a	17.9 ab	35.8 ab	73.0 a	10.3 a

Note: Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range test.

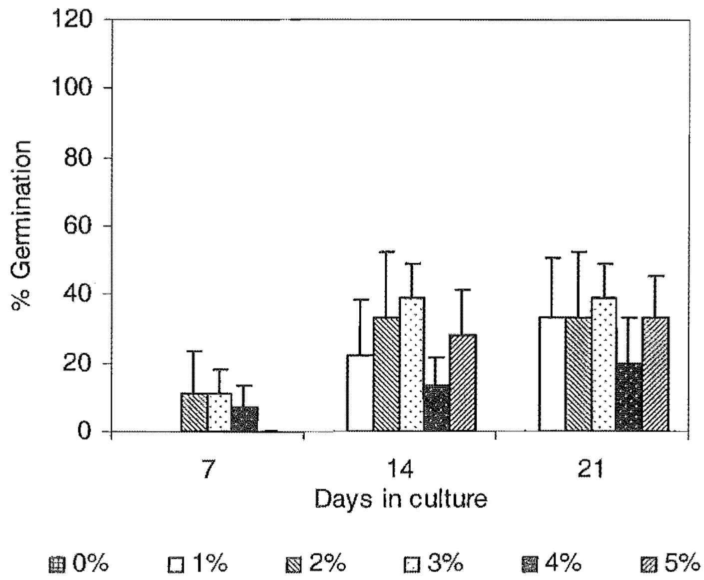


Figure 5. Effect of different maltose concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 5. Effect of different maltose concentrations on the growth of isolated embryos of *Pinus radiata*. All data were obtained at day 21.

Maltose	Mean length (mm)				Mean weight (mg)	
(%)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0	0.0 a	0.0 b	0.0 a	0.0 a	0.0 a	0.0 b
1	4.3 a	7.7 a	1.5 a	13.5 a	30.5 a	2.9 ab
2	3.6 a	7.6 a	1.9 a	13.1 a	27.1 a	2.9 ab
3	5.1 a	6.8 ab	2.4 a	14.3 a	31.3 a	3.4 a
4	2.0 a	2.7 ab	1.1 a	5.9 a	12.0 a	1.4 ab
5	2.9 a	5.0 ab	1.7 a	9.6 a	20.1 a	2.3 ab

Note: Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range test.

3.1.1.3 Organic Nitrogen Sources

A significant effect of the concentrations of L-glutamine on germination percentage of isolated embryos of radiata pine was found ($P<0.05$) (Figure 6). The concentrations of L-glutamine higher than 5.13 mM (i.e. LPSH1 or the basal medium) seemed to have inhibiting effect on the germination of the isolated embryos throughout the study period. However, there was no significant effect of the concentrations of L-glutamine on lengths of cotyledon, hypocotyl and whole plantlet as well as fresh and dry weights. The length of root was affected differently ($P<0.05$) and the root growth seemed to be inhibited when the embryos were cultured on medium with L-glutamine exceeding 5.13 mM (Table 6).

At day 21, although there was no significant effect of concentrations of casein enzymatic hydrolysate on germination percentage (Figure 7), the length of whole plantlet was particularly less in the medium without casein enzymatic hydrolysate (Table 7, $P<0.05$). In addition, casein enzymatic hydrolysate seemed to promote the development of "true" needles by day 21 in the germinated embryos, which was a little bit earlier than those embryos cultured on the medium without the supplement. However, there were no differences in the growth of isolated embryos of *P. radiata* among the treatments with different concentrations of casein enzymatic hydrolysate.

In general, the addition of arginine monohydrochloride to the LPSH1 medium negatively affected both germination percentage ($P<0.01$) and growth ($P<0.05$) of isolated embryos of radiata pine cultured *in vitro* (Figure 8, Table 8).

The addition of polyamines (i.e. putrescine and spermidine) to the LPSH1 medium generally had no or little influence on both germination percentage and growth of isolated embryos of radiata pine cultured *in vitro* (Figures 9 and 10, Tables 9 and 10). The addition of 0.62 μM of putrescine seemed to favour cotyledon elongation but the best hypocotyl growth was observed in the absence of any putrescine in the basal medium ($P<0.05$) (Table 9).

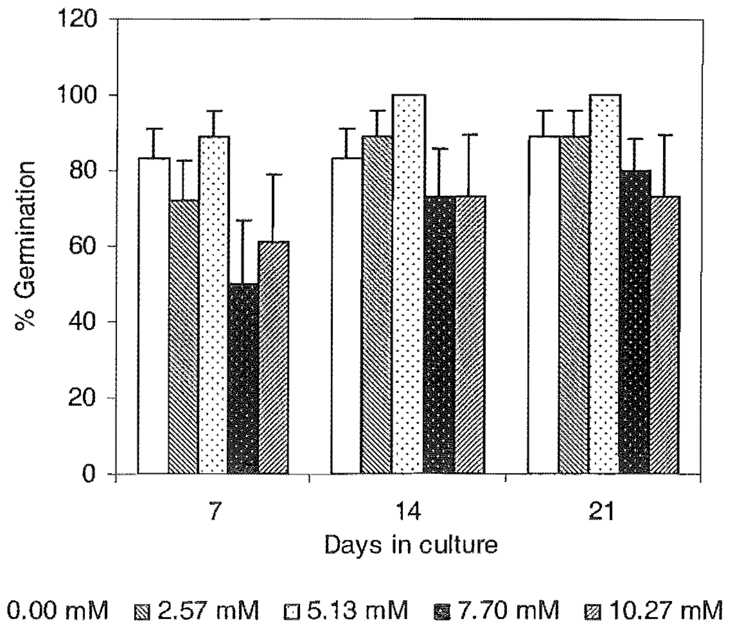


Figure 6. Effect of different glutamine concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 6. Effect of different glutamine concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

Glutamine	Mean length (mm)				Mean weight (mg)	
(mM)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	8.8 a	12.1 a	22.7 a	43.6 ab	68.4 a	8.1 a
2.57	9.6 a	12.2 a	15.7 ab	37.6 ab	65.0 a	8.1 a
5.13	11.9 a	13.8 a	21.4 a	47.2 a	74.5 a	8.8 a
7.70	7.8 a	10.6 a	10.5 b	28.8 b	51.8 a	6.1 a
10.27	11.1 a	13.1 a	11.6 b	35.8 ab	59.4 a	7.3 a

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

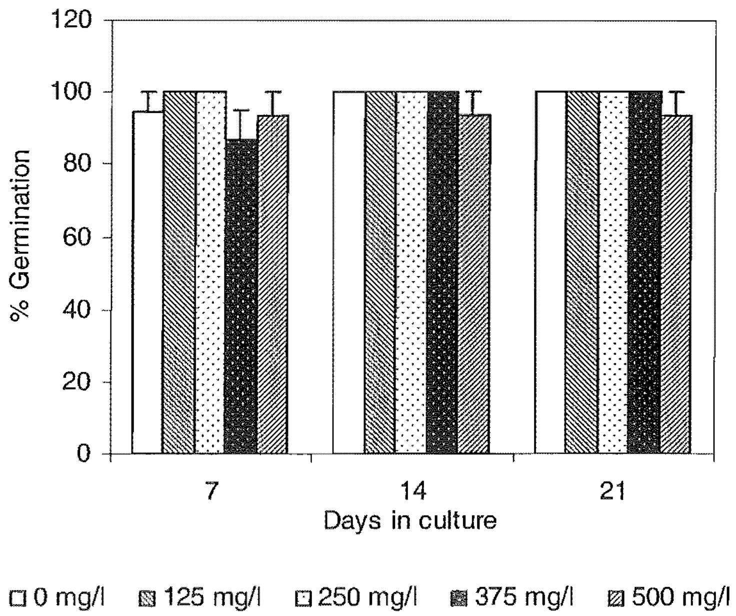


Figure 7. Effect of different casein enzymatic hydrolysate concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 7. Effect of different casein enzymatic hydrolysate concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

Casein enzymatic hydrolysate (mg/L)	Mean length (mm)				Mean weight (mg)	
	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0	10.8 b	12.2 a	14.6 b	37.6 b	67.3 a	8.0 b
125	12.3 ab	13.4 a	20.0 ab	45.7 a	79.5 a	9.0 ab
250	13.7 a	13.7 a	20.9 a	48.2 a	80.6 a	9.5 ab
375	13.3 ab	12.8 a	18.9 ab	45.0 a	80.8 a	9.5 ab
500	12.3 ab	13.6 a	20.1 ab	46.0 a	82.7 a	10.0 a

Note: Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range test.

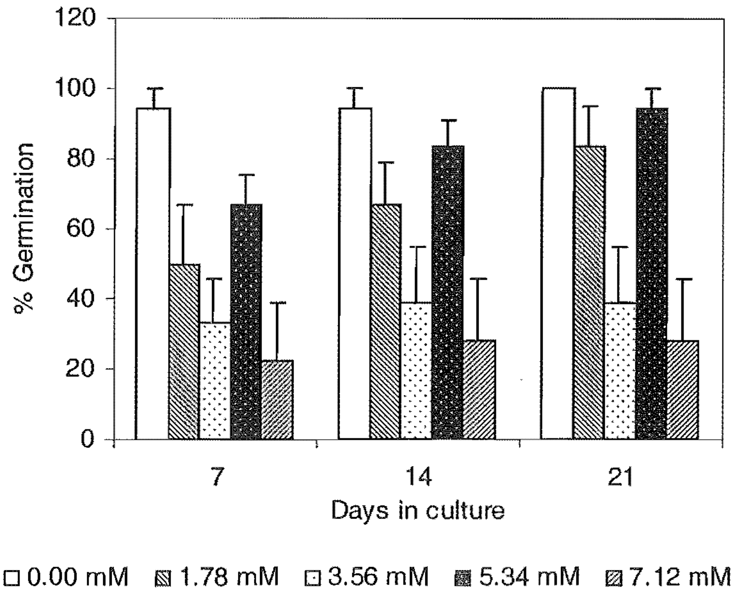


Figure 8. Effect of different concentrations of arginine monohydrochloride on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 8. Effect of different arginine monohydrochloride concentrations on the growth of isolated embryos of *Pinus radiata.* All the data were obtained at day 21.

Arginine monohydro -chloride (mM)	Mean length (mm)				Mean weight (mg)	
	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	10.8 a	17.5 a	16.7 a	45.0 a	66.6 ab	8.1 bc
1.78	9.5 a	14.2 bc	9.5 a	32.9 a	55.7 b	7.7 c
3.56	13.4 a	15.8 ab	10.6 a	39.8 a	73.6 ab	9.8 ab
5.34	13.6 a	15.4 ab	14.8 a	43.8 a	81.1 a	10.2 a
7.12	11.3 a	12.0 c	9.1 a	32.4 a	58.8 b	8.0 bc

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

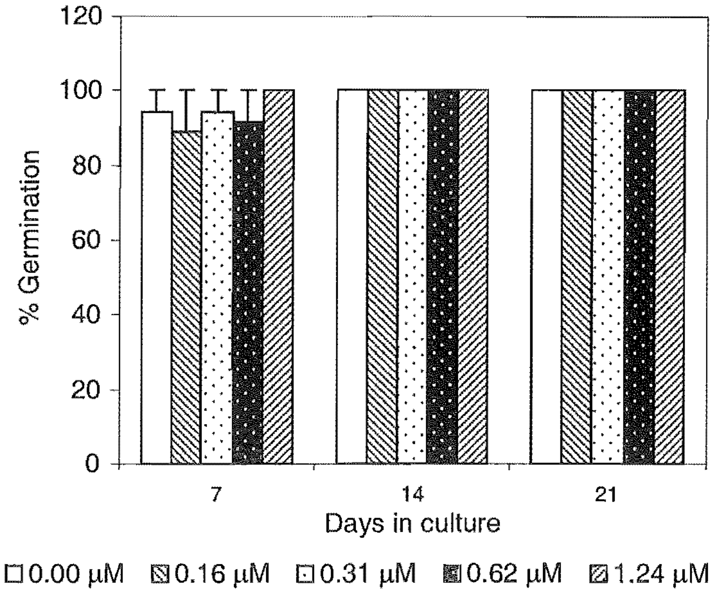


Figure 9. Effect of different putrescine concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 9. Effect of different putrescine concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

Putrescine	Mean length (mm)				Mean weight (mg)	
(µM)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	10.8 c	17.3 a	16.6 a	44.7 a	65.7 a	8.1 a
0.16	11.0 bc	14.2 b	14.7 a	40.0 a	66.5 a	8.5 a
0.31	11.4 bc	14.5 b	19.3 a	45.2 a	65.3 a	8.2 a
0.62	13.5 a	14.6 b	16.3 a	44.4 a	69.0 a	8.4 a
1.24	12.7 ab	15.3 b	19.1 a	47.1 a	70.9 a	8.6 a

Note: Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range test.

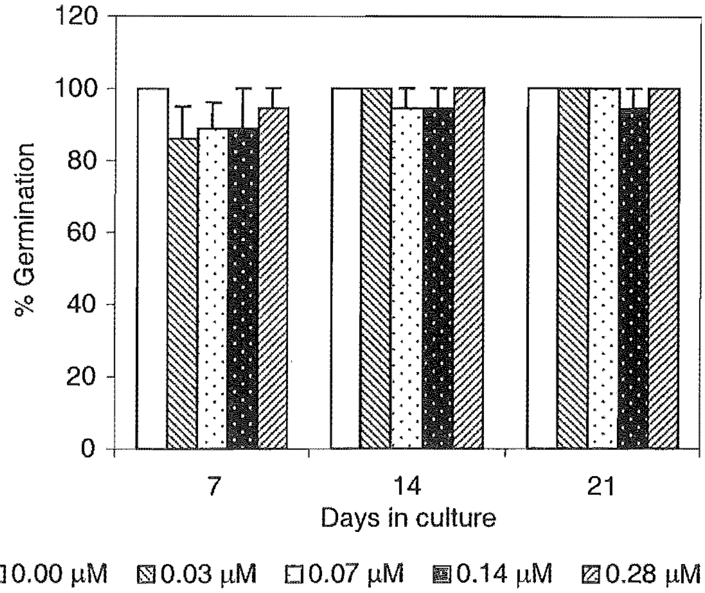


Figure 10. Effect of different spermidine concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 10. Effect of different spermidine concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

Spermidine	Mean length (mm)				Mean weight (mg)	
(µM)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	10.7 a	17.4 a	16.9 a	45.1 a	66.6 a	8.1 a
0.03	10.3 a	16.1 ab	13.3 a	39.7 a	66.8 a	7.8 a
0.07	10.6 a	15.9 ab	14.9 a	41.4 a	66.0 a	7.7 a
0.14	10.2 a	14.9 b	14.7 a	39.8 a	64.5 a	7.7 a
0.28	10.9 a	17.6 a	16.8 a	45.3 a	68.9 a	7.9 a

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

3.1.2 Plant Growth Regulators

3.1.2.1 GA_3 (gibberellic acid)

More isolated embryos of radiata pine germinated in the media containing 0.14-0.58 μM GA_3 at day 7 (Figure 11). After this time, there was little difference in the germination percentage on media with different concentrations of GA_3 tested. However, the growth (i.e. dry weight) of isolated embryo seemed to be reduced slightly by adding GA_3 to the medium ($P<0.05$) (Table 11).

3.1.2.2 Cytokinins

In general, the addition of either BA (6-benzyl adenine) or kinetin to the basal medium had no dramatic effect on the germination percentage of isolated embryos of radiata pine except at day 7 (Figures 12 and13). The growth of the isolated embryos was adversely affected in the presence of any level of cytokinins tested in the basal medium ($P<0.05$) (Tables 12 and 13).

3.1.2.3 Auxins

The addition of IBA (indole-3-butyric acid) or IAA (indole-3-acetic acid) to the basal medium did not have any significant effect on the percentage of germinated isolated embryos of radiata pine (Figures 14 and 15), but significantly reduced the growth of isolated embryos ($P<0.05$) (Tables 14 and 15).

NAA (α-naphthaleneacetic acid) added to the basal medium seems to delay the germination and reduce the growth of isolated embryos of radiata pine. This effect was strongly dependent on the concentrations applied ($P<0.05$) (Figure 16, Table 16). After day 7, the percentage of germination was similar among the different treatments except at the highest NAA concentration tested (0.54 μM), which inhibited germination at all times. Moreover, when cultured on the basal medium supplemented with 0.54 μM NAA, the isolated embryos showed abnormalities, i.e. long and crinkled cotyledons, short and swollen hypocotyl and no root development.

In addition, IAA at all levels tested and NAA from 0.07 to 0.27 μM seemed to have a slightly promotive effect on hypocotyl elongation, but inhibited the root as well as cotyledon growth (Tables 15 and 16).

3.1.2.4 Ethylene

To test the possibility that ethylene could affect the germination and growth of isolated embryos of *P. radiata*, four different concentrations of $AgNO_3$ (silver nitrate, an ethylene action inhibitor) were added to the basal medium. Overall, the addition of $AgNO_3$ to the basal medium had negative effect on germination ($P<0.01$) (Figure 17) and growth of isolated embryos (Table 17) except their dry weights at day 21.

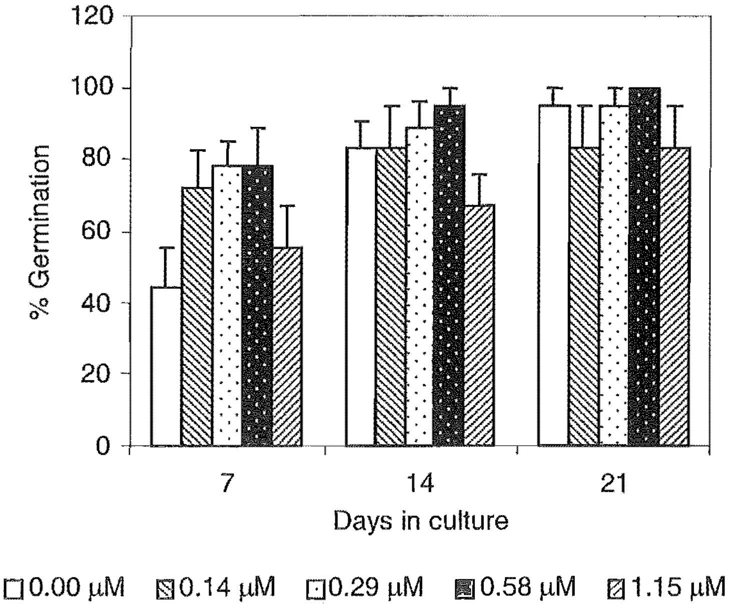


Figure 11. Effect of different GA_3 concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 11. Effect of different GA_3 concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

GA_3	Mean length (mm)				Mean weight (mg)	
(µM)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	10.9 a	12.1 a	22.7 a	45.7 a	80.8 a	10.3 a
0.14	10.4 a	12.5 a	14.8 a	37.6 a	75.6 ab	9.1 b
0.29	9.6 a	12.8 a	15.9 a	38.3 a	63.6 b	8.2 b
0.58	11.8 a	12.7 a	17.2 a	41.6 a	69.8 ab	8.7 b
1.15	9.8 a	12.3 a	16.0 a	38.1 a	62.3 b	8.0 b

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

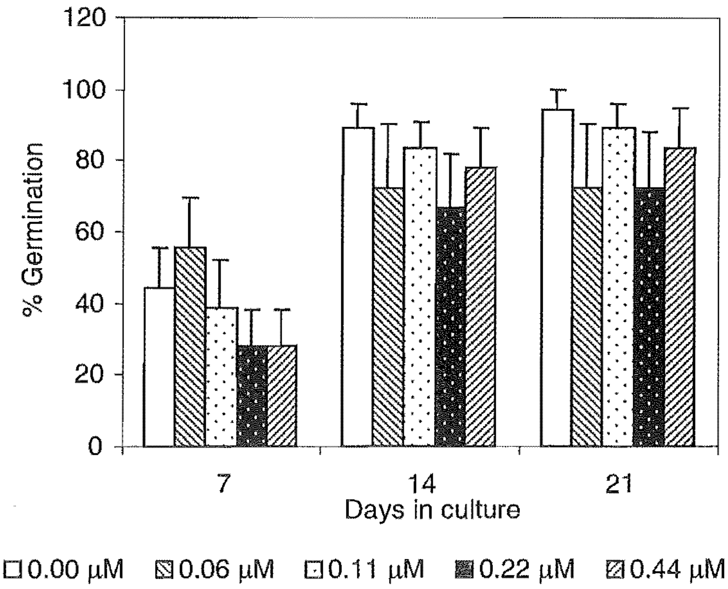


Figure 12. Effect of different BA concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 12. Effect of BA concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

BA	Mean length (mm)				Mean weight (mg)	
(µM)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	11.5 a	12.4 a	22.7 a	46.6 a	81.0 a	10.5 a
0.06	9.4 ab	12.2 a	10.6 b	32.2 b	61.2 b	7.4 b
0.11	7.7 b	10.4 ab	9.0 b	27.1 bc	57.8 b	7.2 b
0.22	8.2 b	10.6 ab	9.0 b	27.8 bc	57.5 b	7.3 b
0.44	7.5 b	9.2 b	6.5 b	23.2 c	56.9 b	7.2 b

Note: Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range test.

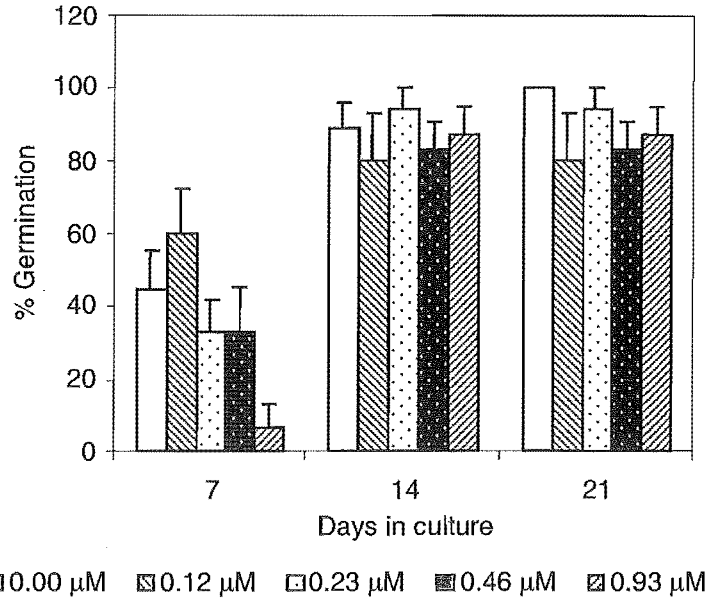


Figure 13. Effect of different kinetin concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 13. Effect of different kinetin concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

Kinetin	Mean length (mm)				Mean weight (mg)	
(µM)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	11.1 a	12.4 a	22.8 a	46.4 a	80.5 a	10.3 a
0.12	8.8 ab	11.9 a	12.8 b	33.5 b	48.8 b	6.4 b
0.23	6.2 b	11.3 a	8.6 b	26.1 b	47.0 b	6.4 b
0.46	6.9 b	12.6 a	8.4 b	27.9 b	52.4 b	6.7 b
0.93	7.6 b	11.4 a	8.0 b	27.0 b	54.1 b	6.8 b

Note: Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range test.

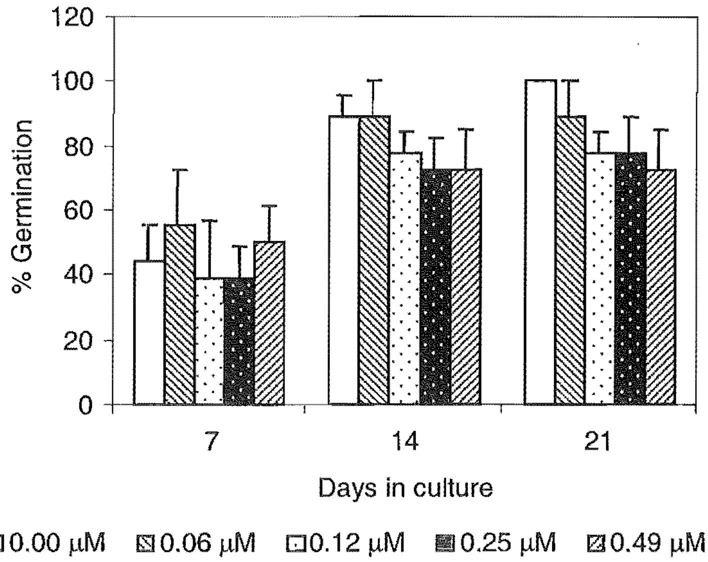


Figure 14. Effect of different IBA concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 14. Effect of different IBA concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

IBA	Mean length (mm)				Mean weight (mg)	
(µM)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	11.2 a	12.2 a	22.5 a	45.9 a	80.7 a	10.3 a
0.06	5.5 c	13.3 a	15.4 b	34.2 b	71.9 ab	9.0 b
0.12	8.7 b	13.1 a	10.9 bc	32.6 b	69.0 ab	8.7 b
0.25	7.2 bc	11.3 a	8.8 bc	27.3 b	63.9 b	8.3 b
0.49	8.4 b	13.8 a	7.3 c	29.6 b	62.9 b	8.3 b

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

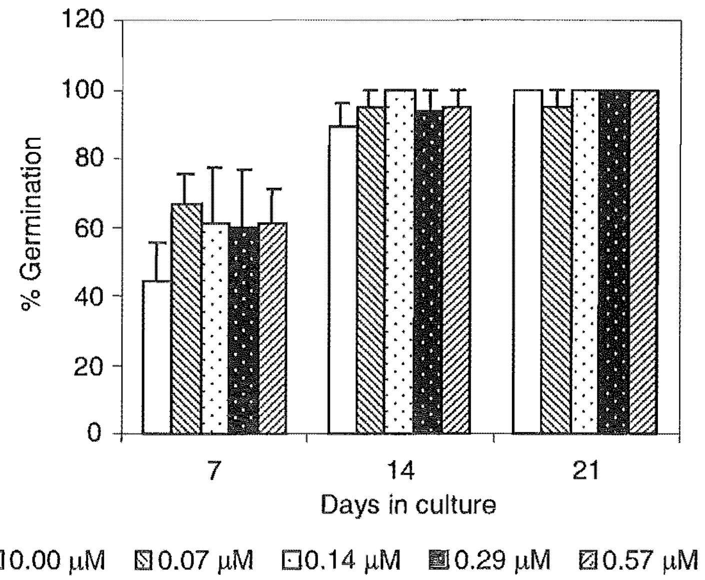


Figure 15. Effect of different IAA concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 15. Effect of different IAA concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

IAA	Mean length (mm)				Mean weight (mg)	
(µM)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	11.1 a	12.2 b	20.7 a	44.0 a	80.7 a	10.3 a
0.07	8.5 a	13.9 ab	12.1 b	34.5 b	58.1 b	8.1 b
0.14	10.9 a	14.4 a	15.4 ab	40.7 ab	64.2 ab	8.5 b
0.29	10.6 a	13.5 ab	15.3 ab	39.3 ab	65.9 ab	9.2 ab
0.57	10.1 a	14.4 a	15.2 ab	39.7 ab	63.8 ab	8.6 b

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

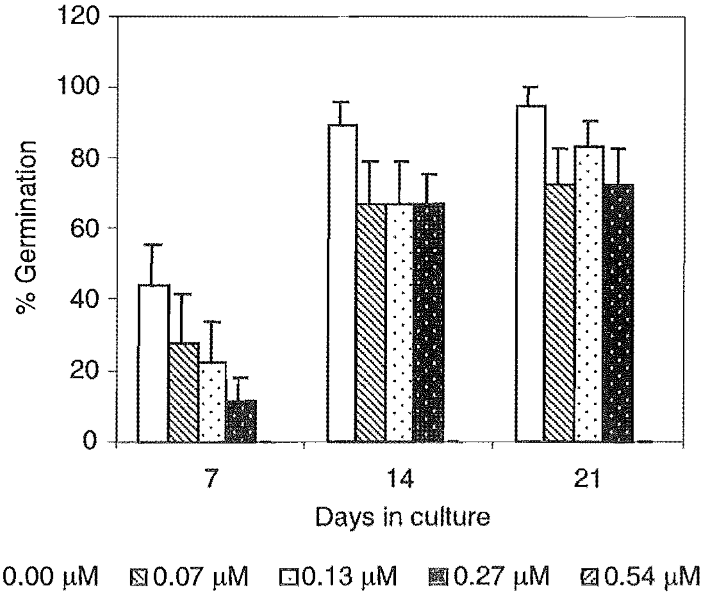


Figure 16. Effect of different NAA concentrations on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 16. Effect of different NAA concentrations on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

NAA	Mean length (mm)				Mean weight (mg)	
(μM)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	11.6 b	12.2 ab	22.4 a	46.2 a	80.7 ab	10.4 a
0.07	7.6 b	13.9 a	10.3 b	31.9 b	75.2 ab	9.3 a
0.13	8.3 b	14.7 a	7.8 b	30.8 b	71.2 b	8.9 a
0.27	10.2 b	15.0 a	5.5 bc	30.7 b	92.4 a	10.2 a
0.54	19.7 a	9.6 b	1.0 c	30.3 b	86.2 ab	9.3 a

Note: Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range test.

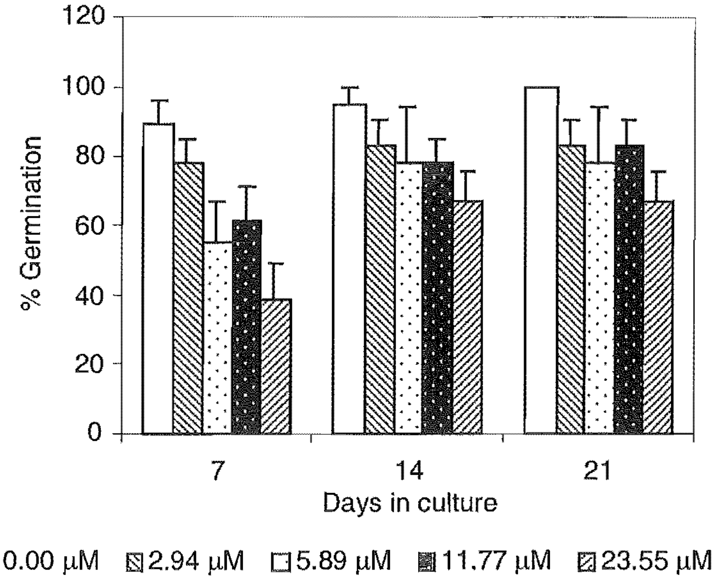


Figure 17. Effect of different concentrations of $AgNO_3$ (an ethylene action inhibitor) on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 17. Effect different concentrations of $AgNO_3$ (an ethylene action inhibitor) on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

$AgNO_3$	Mean length (mm)				Mean weight (mg)	
(µM)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0.00	11.3 a	17.5 a	16.8 a	45.6 a	66.6 a	8.1 a
2.94	8.9 ab	13.9 bc	10.4 b	33.3 b	60.1 ab	7.7 a
5.89	8.1 b	12.1 dc	6.9 c	27.1 c	51.7 b	7.1 a
11.77	6.9 b	11.7 d	7.2 c	25.8 c	57.0 ab	7.7 a
23.55	6.1 b	14.2 b	7.6 bc	27.9 c	52.6 b	6.9 a

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

3.1.3 Physical Factors

3.1.3.1 Different Ways of Placing Embryos on Medium

The three different ways of sowing embryos tested did not have any adverse effect on germination percentage of the isolated embryos (Figure 18). However, the growth of isolated embryos was apparently affected by how the isolated embryos were placed on the medium (Table 18). The best root growth was observed when the cotyledons were embedded into the medium (Table 18, Plate 2).

3.1.3.2 Agar-gelled and Liquid Media

Isolated embryos of radiata pine cultured in liquid medium showed significantly better growth ($P<0.01$) (Plate 3, Table 19), but lower germination percentage compared to liquid with sponge or 0.8% agar-gelled medium ($P<0.01$) (Table 19, Figure 19). However, if isolated embryos were cultured in a larger volume of liquid medium, some embryos that germinated normally would become purple and finally died once they were totally under the surface of the liquid medium (Plate 4). The growth of the isolated embryos was least in the liquid with sponge treatment (Table 19).

3.1.3.3 PEG (polyethylene glycol)

To investigate further the potential of liquid medium for the conversion of embryos into plantlets, the effect of PEG 6,000 added to the liquid medium (i.e. LPSH1 or basal medium) was studied. 1 or 3 % of PEG could increase significantly the percentage of germinated isolated embryos ($P<0.05$) but had no negative effect on the growth of the isolated embryos. (Figure 20, Table 20). The two other higher PEG concentrations had little effect on germination and growth of the isolated embryos of radiata pine.

3.1.3.4 Light

Germination of isolated embryos of radiata pine appeared to be independent of light (Figure 21). In contrast, the growth of the isolated embryos could be influenced significantly by light in different ways ($P<0.01$) (Table 21). Among the three treatments, 16-hour photoperiod appeared to be the best for root growth, but for cotyledon development continuous light condition seemed to be the best. In continuous darkness, the hypocotyl appeared to elongate more, but the cotyledon and root did not fare well with the two other treatments.

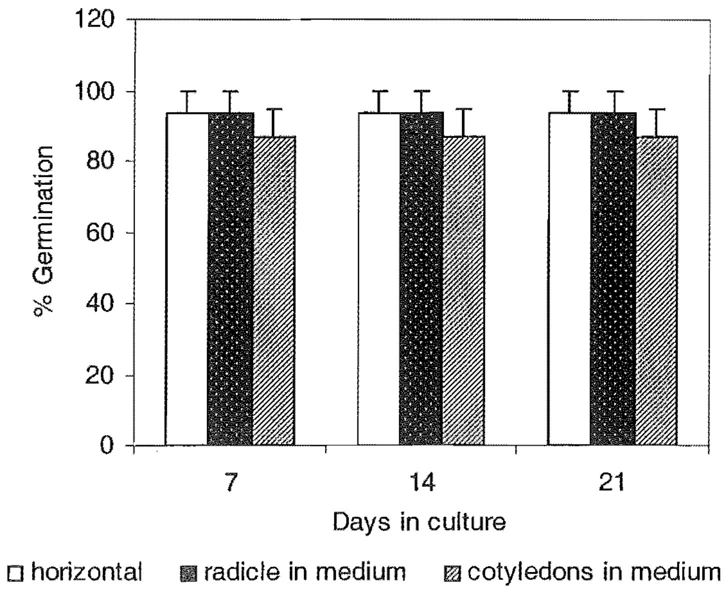


Figure 18. Effect of different ways of sowing isolated embryos of *Pinus radiata* on mean germination percentage over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 18 Effect of different ways of sowing isolated embryos of *Pinus radiata* on the growth of embryos. All data were obtained at day 21.

Treatment	Mean length (mm)				Mean weight (mg)	
	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
Horizontal	11.9 a	16.0 a	24.7 ab	52.7 ab	73.4 a	9.1 b
Radicle in medium	10.3 a	16.1 a	20.2 b	46.5 b	55.2 b	7.0 b
Cotyledons in medium	11.6 a	18.4 a	35.0 a	65.0 a	87.2 a	16.0 a

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

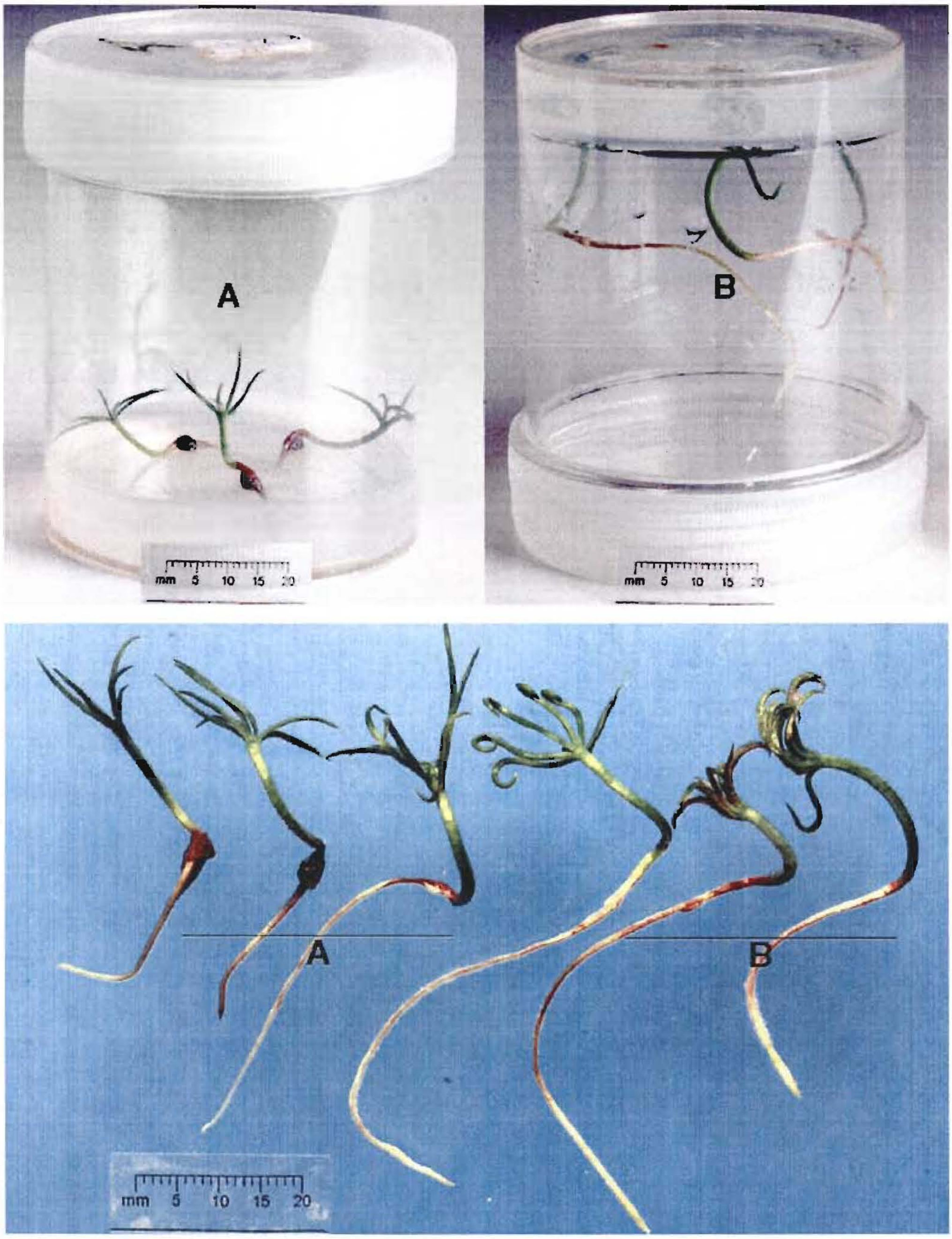


Plate 2. Isolated embryos of *Pinus radiata* placed horizontally on the surface of the medium (A), or with the cotyledons embedded in medium and root in air (B). The photos were taken after 21 days of culture.

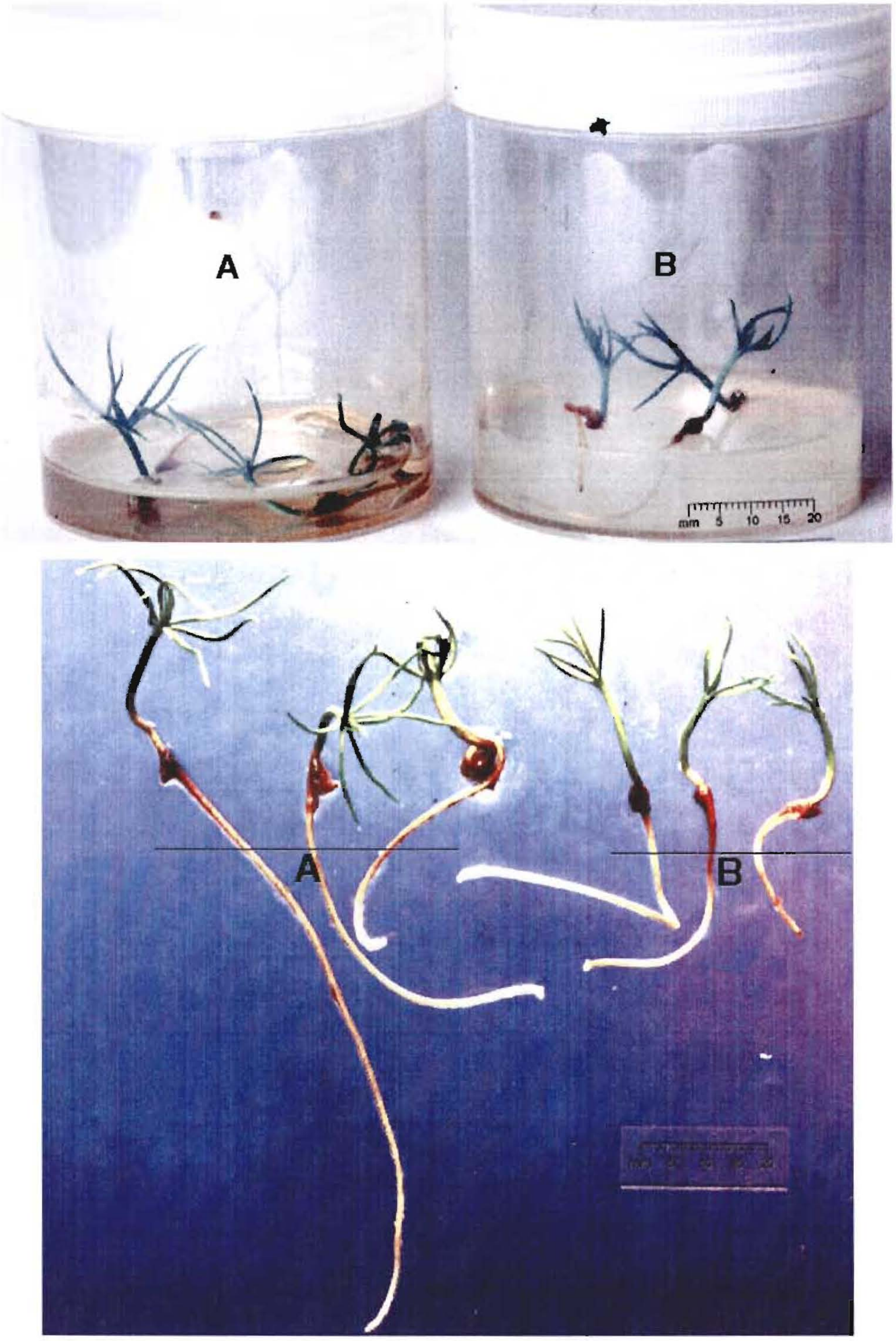


Plate 3. Isolated embryos of *Pinus radiata* cultured on liquid medium (ca. 6 mm thick) (A), or 0.8% (w/v) agar-gelled medium (B). The photos were taken after 21 days of culture.

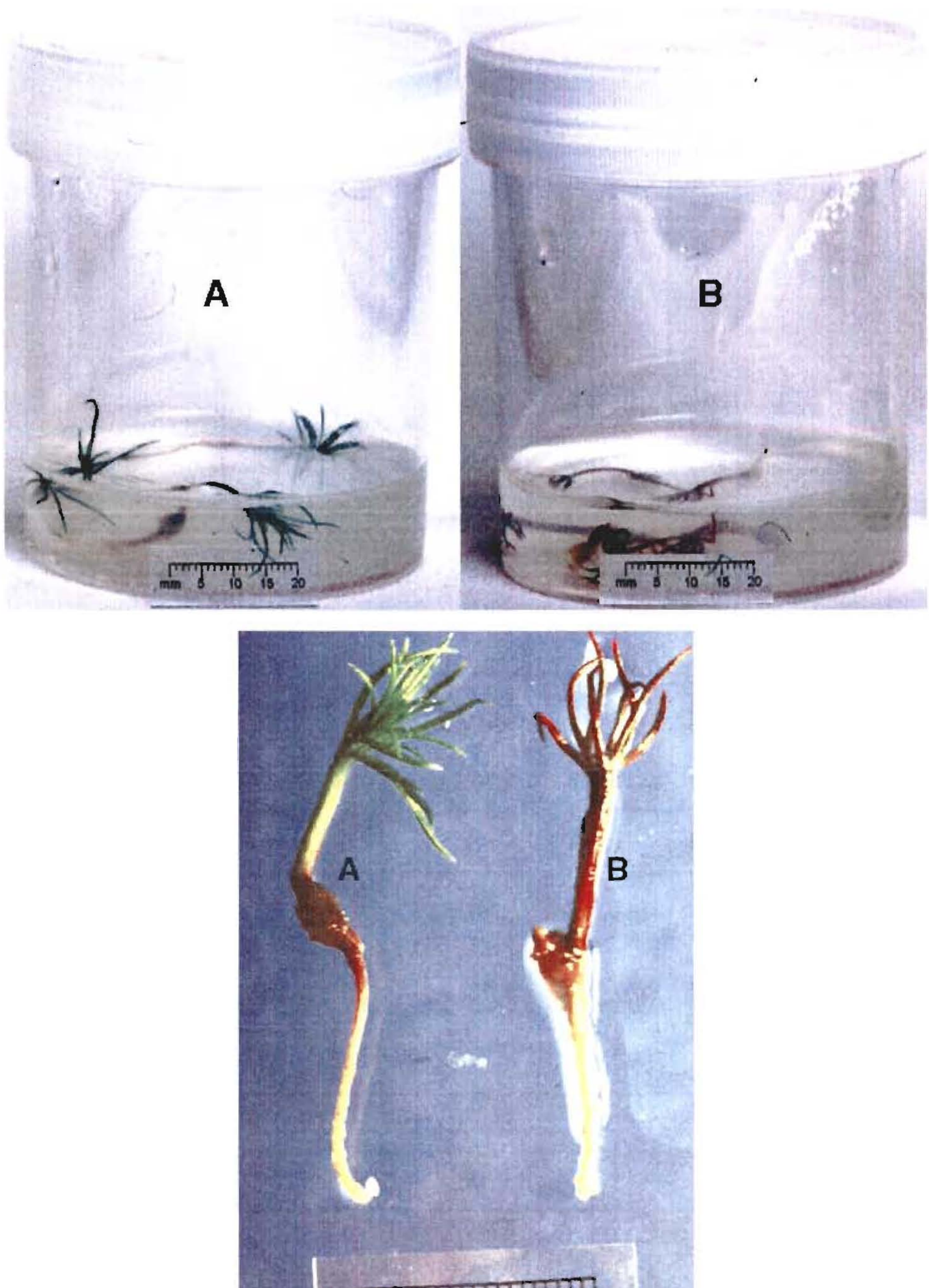


Plate 4. Isolated embryos of *Pinus radiata* cultured on the same volume of normally used liquid medium (ca. 12 mm thick). A, green alive plantlet; B, purple dead plantlet. The photos were taken after 21 days of culture.

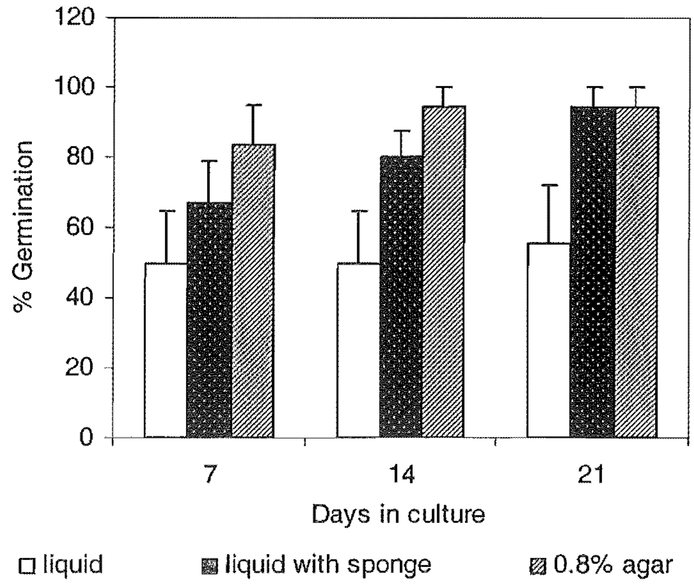


Figure 19. Effect of agar-gelled and liquid media on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 19. Effect of agar-gelled and liquid media on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

Treatment	Mean length (mm)				Mean weight (mg)	
	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
Liquid	17.6 a	16.8 a	23.0 a	57.3 a	109.0 a	16.0 a
Liquid with sponge	7.3 c	12.1 b	7.5 b	26.9 c	38.4 c	5.8 c
0.8% agar	11.8 b	14.8 a	19.2 a	45.8 b	72.4 b	8.8 b

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

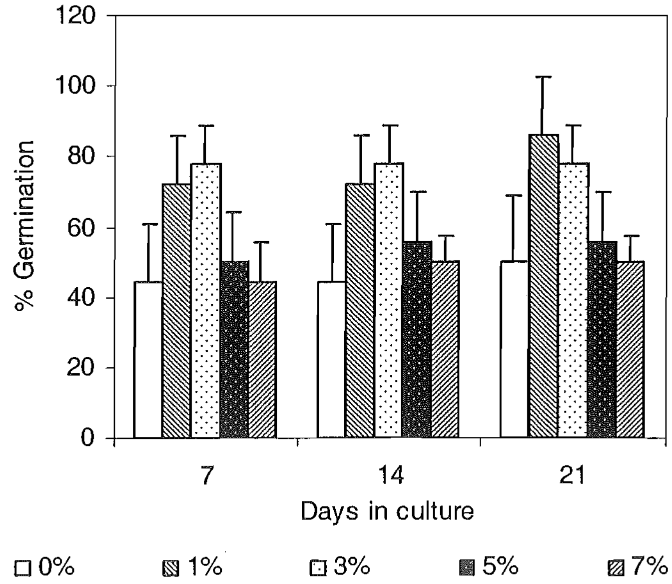


Figure 20. Effect different concentrations of PEG 6,000 on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 20. Effect different concentrations of PEG 6,000 on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

PEG	Mean length (mm)				Mean weight (mg)	
(%)	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
0	17.4 a	17.0 a	23.3 a	57.8 ab	109.1 a	16.0 ab
1	14.8 ab	18.2 a	23.8 a	56.8 ab	102.6 a	14.7 ab
3	15.1 ab	18.9 a	35.1 a	69.1 a	110.7 a	18.9 a
5	10.9 b	16.6 a	29.5 a	57.0 ab	111.1 a	20.0 a
7	12.7 b	12.8 b	25.0 a	50.4 b	96.4 a	12.3 b

Note: Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range test.

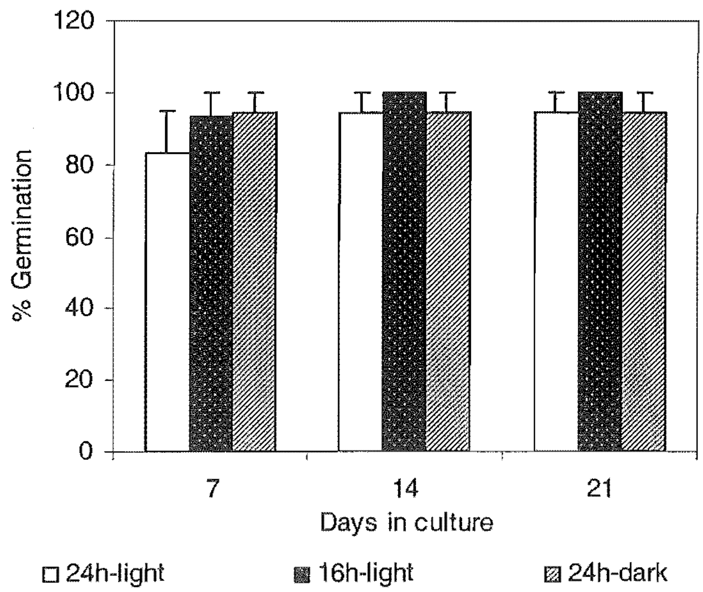


Figure 21. Effect of light on mean germination percentage of isolated embryos of *Pinus radiata* over time under *in vitro* conditions. Error bars indicate the standard error of the mean.

Table 21. Effect of light on the growth of isolated embryos of *Pinus radiata.* All data were obtained at day 21.

Treatment	Mean length (mm)				Mean weight (mg)	
	Cotyledon	Hypocotyl	Root	Total	Fresh	Dry
24h-light	11.8 a	14.8 b	19.2 b	45.8 a	72.4 ab	8.8 a
16h-light	9.4 b	10.9 c	29.7 a	50.0 a	62.4 b	8.3 a
24h-dark	5.1 c	29.3 a	15.9 b	50.3 a	80.2 a	7.2 b

Note: Different letters within a column indicate significant differences at $P = 0.05$ by Duncan's multiple range test.

3.2 Seed Germination Test

To verify if seedcoat could affect seed germination of *P. radiata* three treatments were tested in this experiment. The hard seedcoat strongly restricts germination of *P. radiata* seeds (Figure 22, Plate 5). At day 7, only 8.3% of the intact seeds germinated, which was significantly (P<0.01) lower than those with their hard seedcoat removed (82.5%). By day 21 the percent germination of intact seeds was still substantially lower (41.7%) in this experiment. However, most of the ungerminated intact seeds could germinate within one week once the hard seedcoat was removed. Contrary to hard seedcoat, the soft seedcoat appeared to be necessary for seed germination of *P. radiata*. Although the percent germination of seeds without hard and soft seedcoats was considerably (P<0.01) higher than that of the intact seeds at day 7, by day 21 there was no difference between these two treatments. Furthermore, the megagametophytes of most ungerminated seeds without hard and soft seedcoats had become visibly rotten after two weeks.

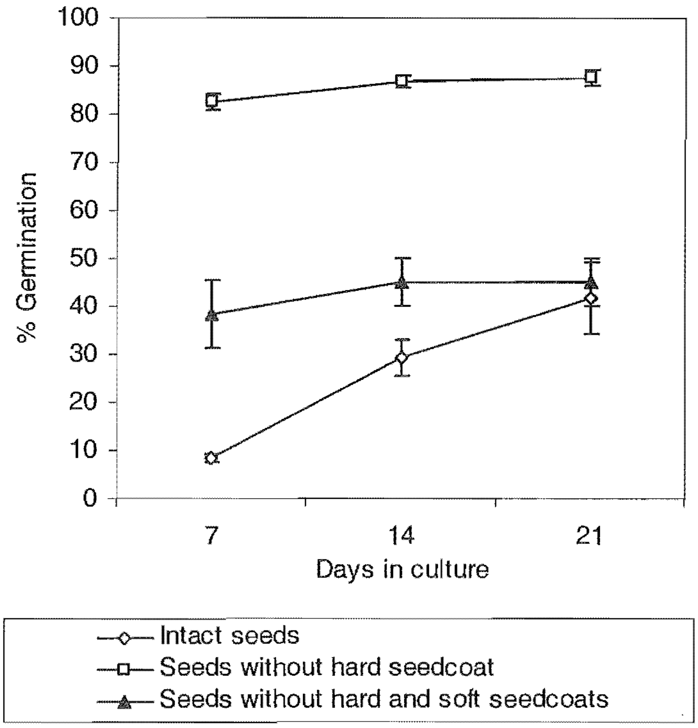


Figure 22. Effect of seedcoats removal on mean germination percentage of *Pinus radiata* seeds. Error bars indicate the standard error of the mean.

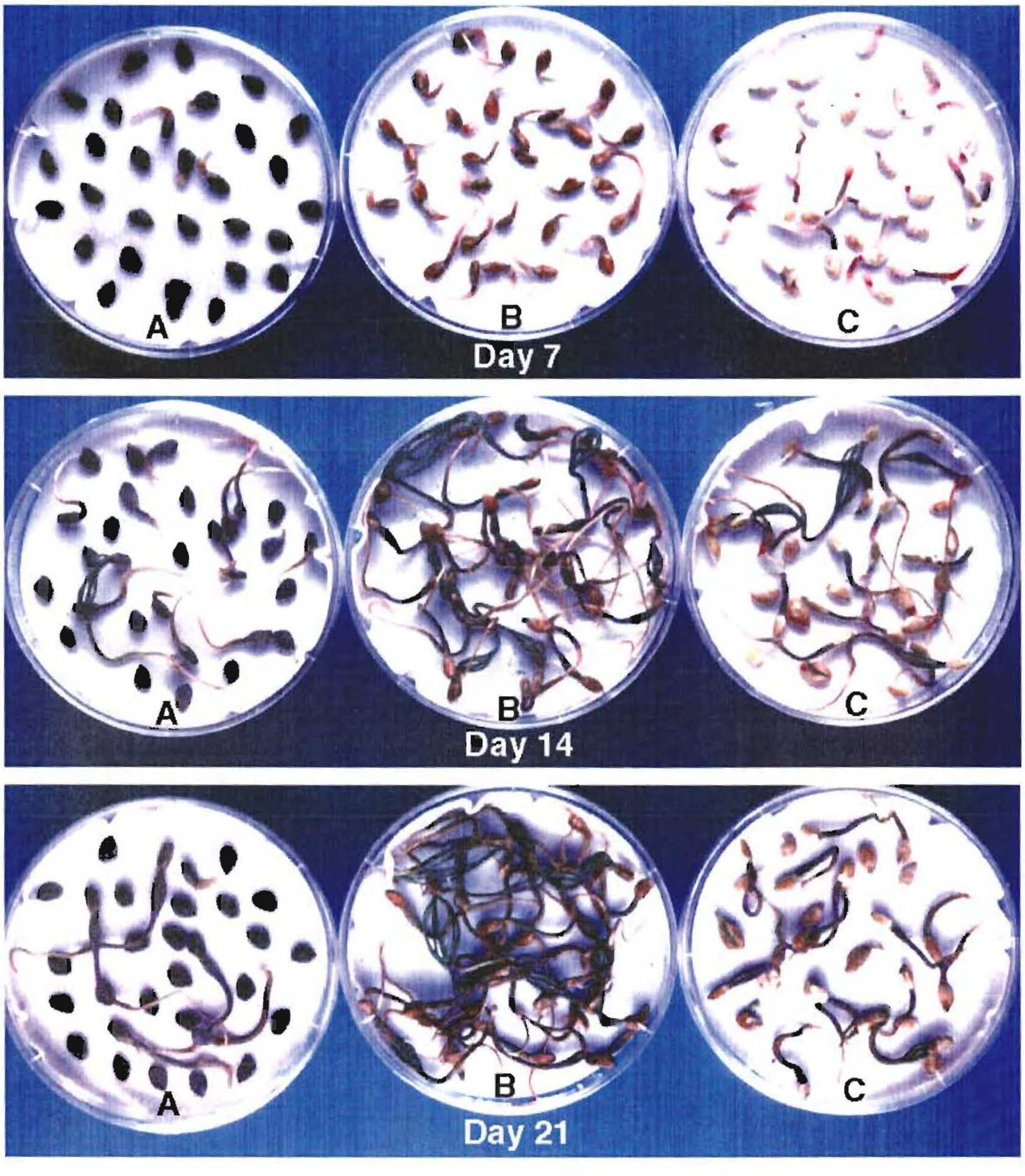


Plate 5. Effect of seedcoats on germination of *Pinus radiata* seeds.

A: Intact seeds.

B: Seeds without hard seedcoat.

C: Seeds without both hard and soft (inner papery membrane) seedcoats.

3.3 Performance of Isolated Embryos Cultured *in Vitro* — A Comparative Study

3.3.1 Isolated Embryos Cultured on Optimum Medium (LPSH2)

3.3.1.1 Changes of Length

The patterns of changes in the lengths of the different parts of the emblings (i.e. the plantlets developed from isolated embryos cultured on LPSH2 medium) were significantly different from those of the seedlings (i.e. the plantlets developed from seeds without hard seedcoat and cultured on water agar medium) ($P<0.01$) (Figure 23a-c). At day 7, the lengths of cotyledon and hypocotyl were similar between emblings and seedlings (Figure 23a and b, Plate 6). However, the root of seedlings was much longer than that of emblings (Figure 23c, Plate 6). From day 7 to day 14, the length of seedling and its various parts increased sharply, whereas the length of embling and that of its parts increased slightly during this time. Therefore, by day 14 the length of seedlings was nearly three times longer than that of emblings (Figure 23a-d).

3.3.1.2 Changes of Fresh Weight

Similar to the changes of lengths, the patterns of fresh weight changes were also significantly different between emblings /seedlings and their different parts ($P<0.01$) (Figure 24a-d).

3.3.2 Isolated Embryos Cultured on 0.8% (w/v) Water Agar Medium

No root development was observed when isolated embryos cultured on the medium without external supply of nutrients, i.e. 0.8% (w/v) water agar medium (Plate 7). The resultant plantlets (i.e. water agar emblings) were significantly smaller than those cultured on LPSH2, the optimum nutrient medium (Figure 23d and 24d).

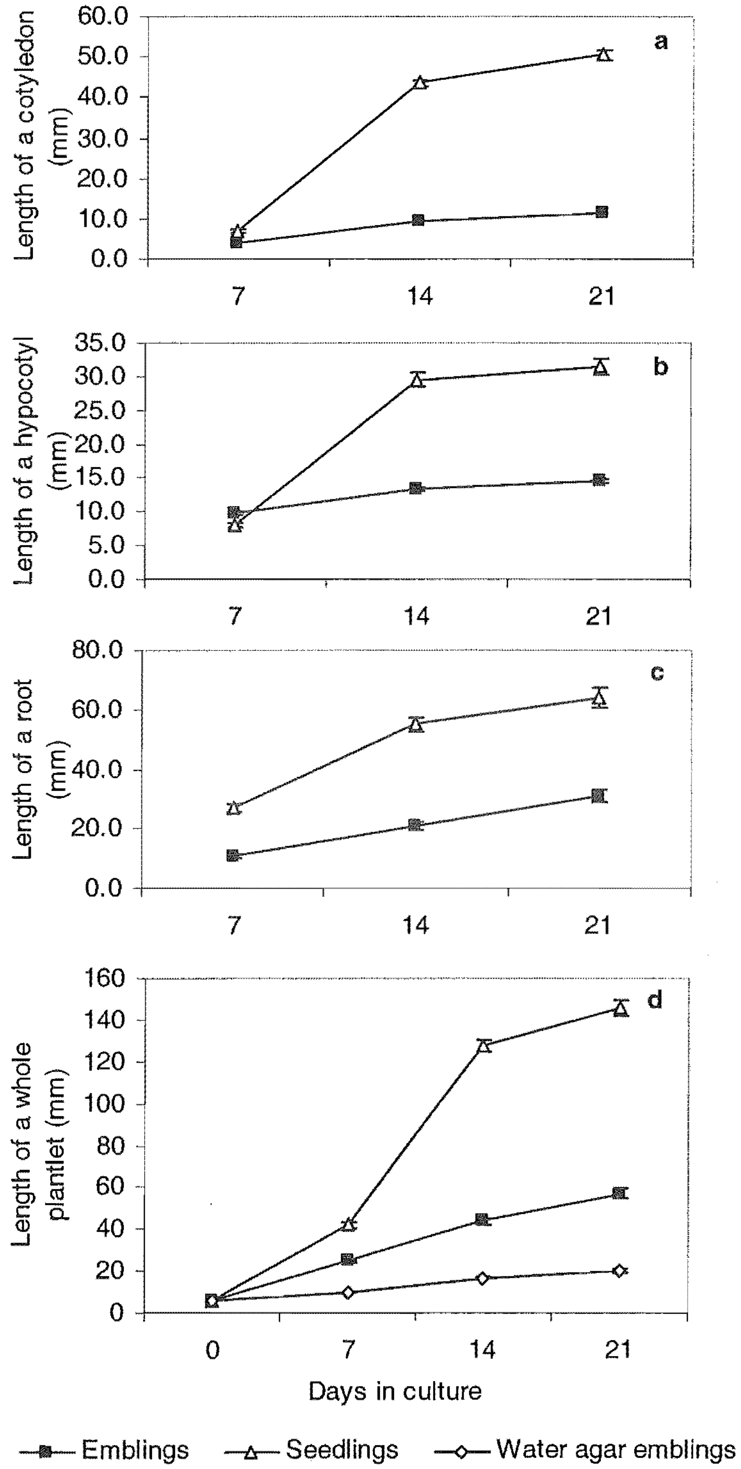


Figure 23. Time course of changes in mean lengths of various parts of emblings /seedlings of *Pinus radiata*. Error bars indicate the standard error of the mean and some are less than the size of a symbol.

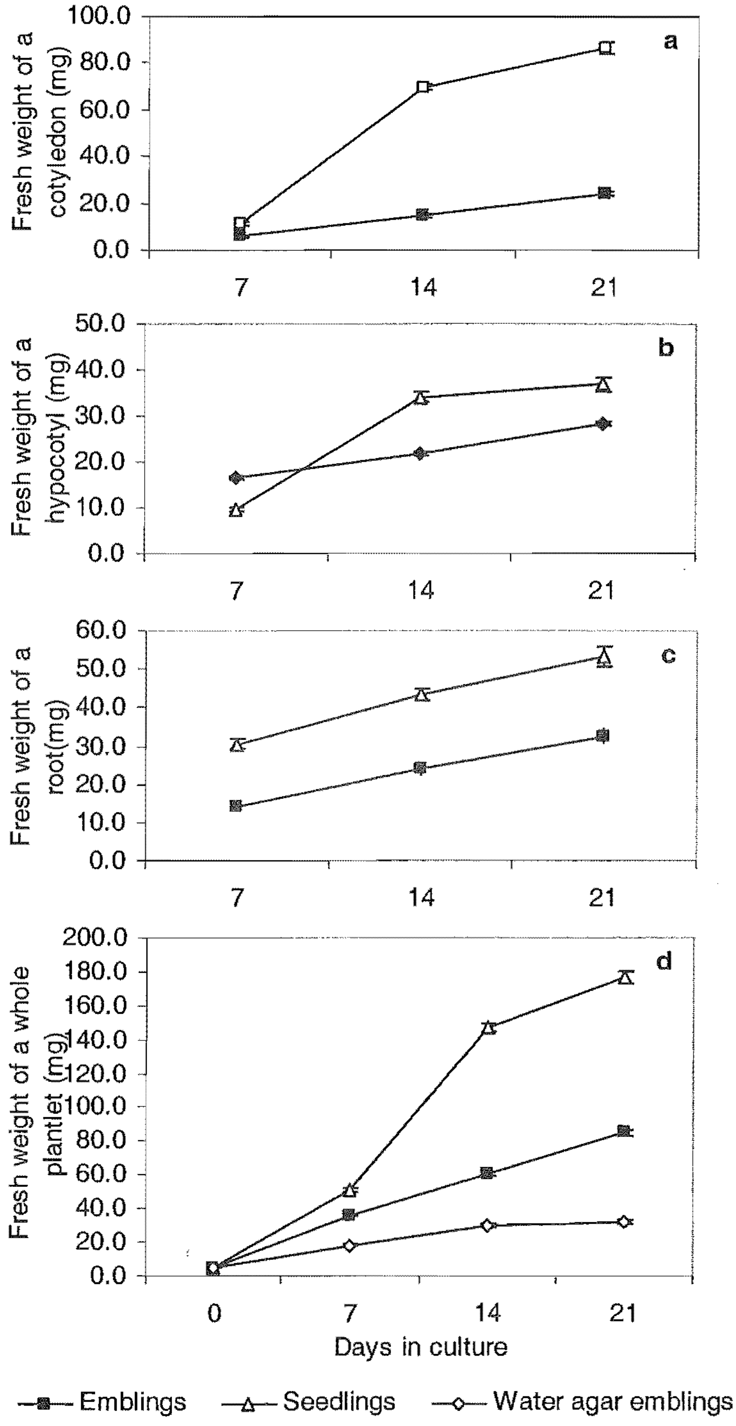
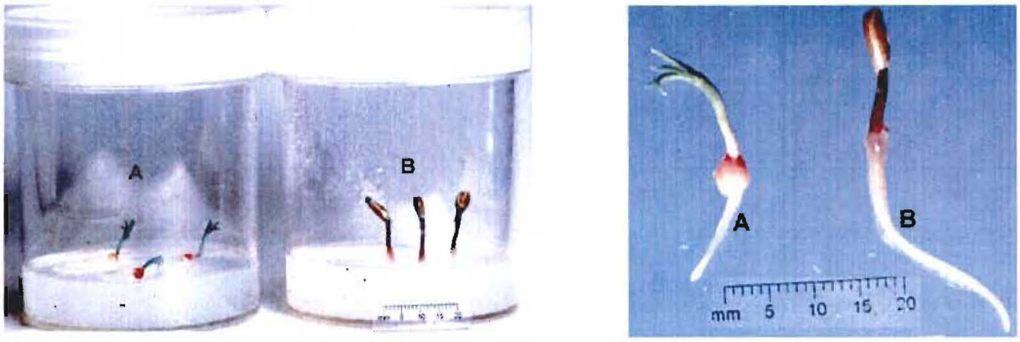
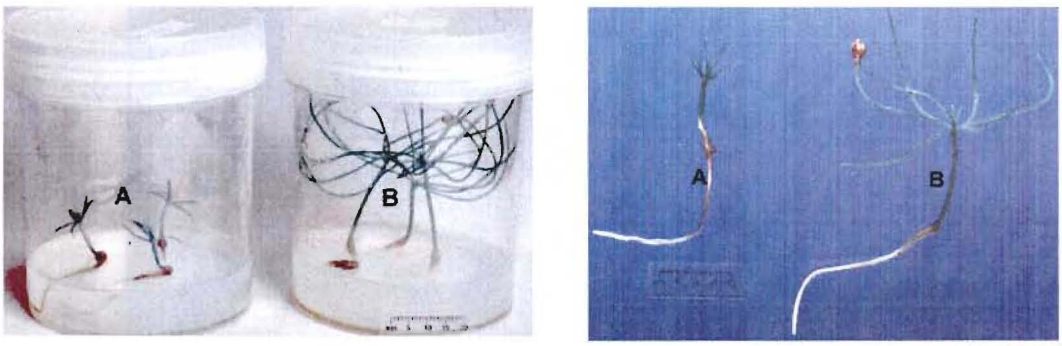


Figure 24. Time course of changes in mean fresh weights of various parts of emblings /seedlings of *Pinus radiata*. Error bars indicate the standard error of the mean and some are less than the size of a symbol.



Day 7

Day 14



Day 21

Plate 6. Performance of *Pinus radiata* isolated embryos cultured on LPSH2 medium (A) and that of seeds without hard seedcoat cultured on water agar medium (B).

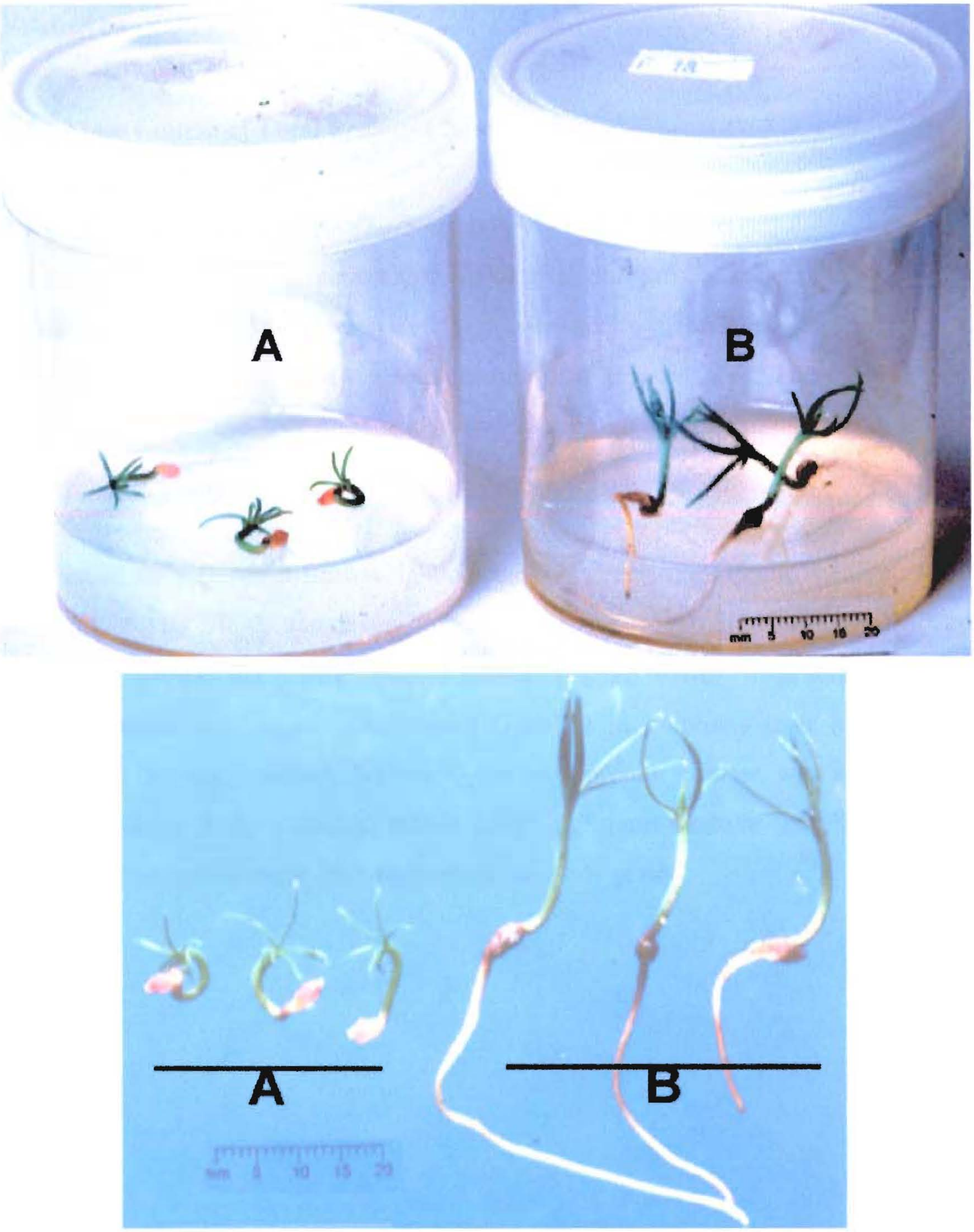


Plate 7. Isolated embryos of *P. radiata* cultured on water agar medium (A), or LPSH2 medium (B). The photos were taken after 21 days of culture.

3.4 Biochemical Changes Associated with the Conversion of Isolated Embryos of *P. radiata*

3.4.1 Time Course of Total Protein Changes

3.4.1.1 Protein Concentration Determination

On fresh weight basis, the patterns of changes in total protein concentrations in the cotyledons were similar between emblings and seedlings, both of them decreasing rapidly from day 7 to day 14 before levelling off (Figure 25a). The total protein concentrations in the hypocotyl and root of emblings and seedlings were similar throughout the study period (Figure 25b and c).

Because of the patterns of fresh weight changes between emblings and seedlings were different (Figure 24a-d), the changes in total protein concentration were recalculated as μg total protein per plantlet part or plantlet (Figure 26a-d). In general, the total protein content was significantly lower ($P<0.01$) in emblings than in seedlings throughout the study period. However, the total protein content was significantly higher ($P<0.01$) in the emblings grown on the optimum medium (LPSH2) than on water agar medium (Figure 26d) throughout the study period.

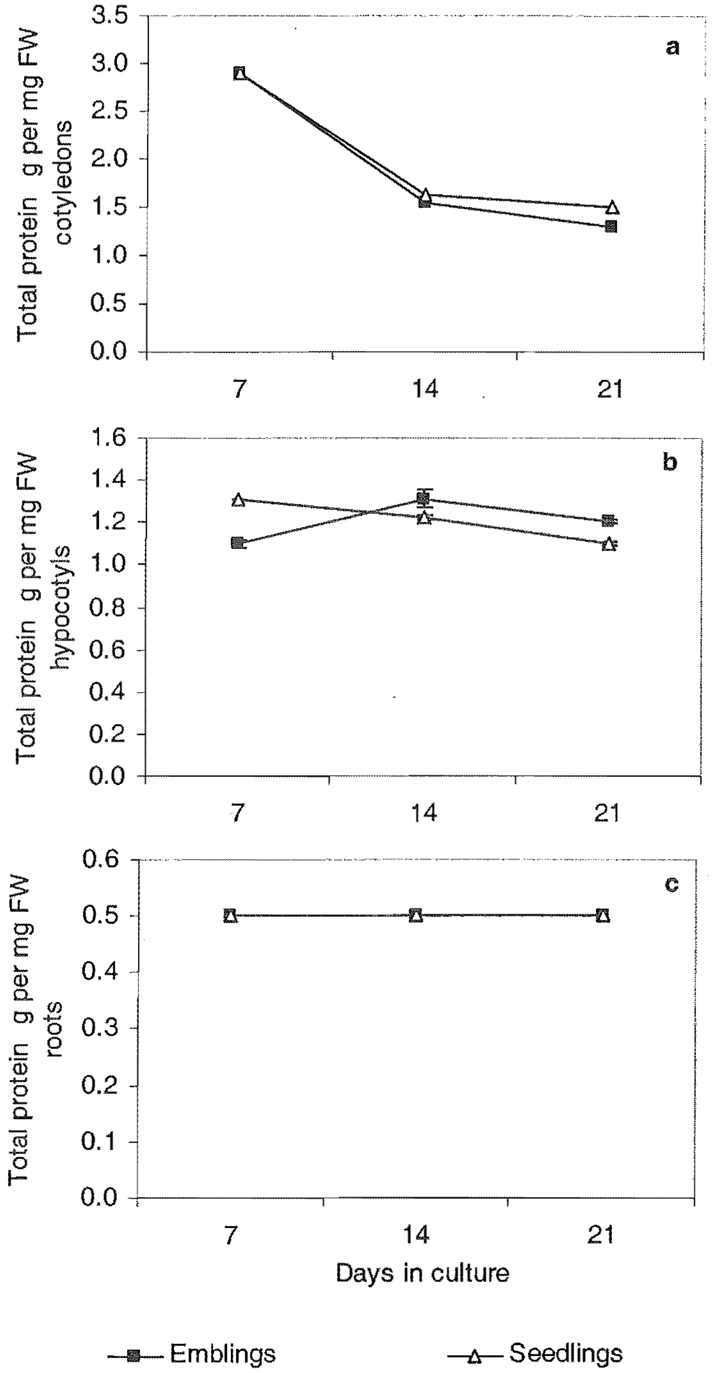


Figure 25. Time course of changes in mean total protein concentrations (μg per mg fresh weight tissue) in the various parts of emblings /seedlings of *Pinus radiata.* Error bars indicate the standard error of the mean and some are less than the size of a symbol.

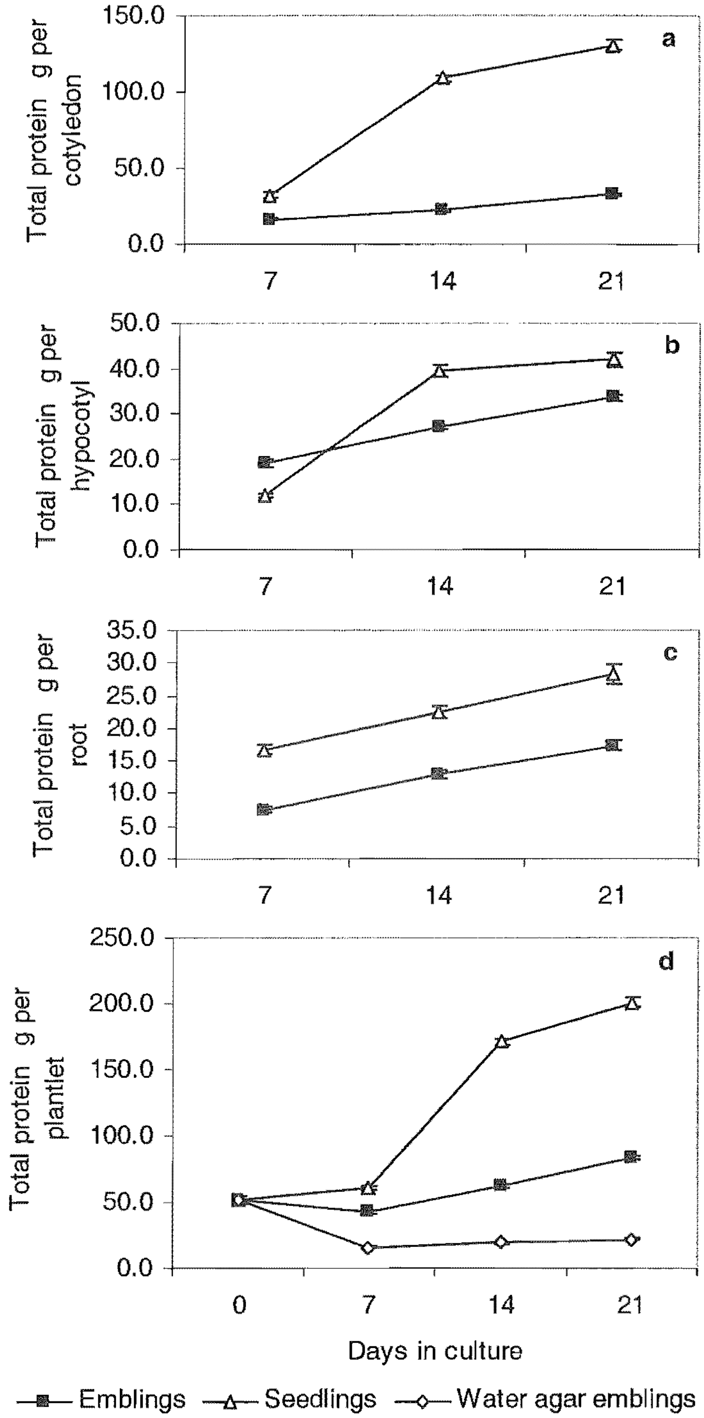


Figure 26. Time course of changes in mean total protein concentrations of various seed parts or plantlets of *Pinus radiata*. Error bars indicate the standard error of the mean and some are less than the size of a symbol.

3.4.1.2 SDS-PAGE of Proteins

Little or no noticeable difference in the protein profiles after SDS-PAGE was observed between emblings. and seedlings throughout the whole study period (Plate 8). The patterns of bands were significantly different between embryos (day 0) and the various seed parts from day 7 to day 21. In the cotyledons, several major polypeptides that were present at day 7 were absent or underexpressed at day 14 and day 21, particularly those with molecular weights of around 66.2-97.4 kDa (Plate 8A). The same patterns of protein changes was displayed when the whole emblings grown on water agar were studied (Plate 8D). Contrary to the cotyledons or the emblings grown on water agar, the protein profiles of both the hypocotyl and root did not appear to change over 21 days (Plate 8B and C).

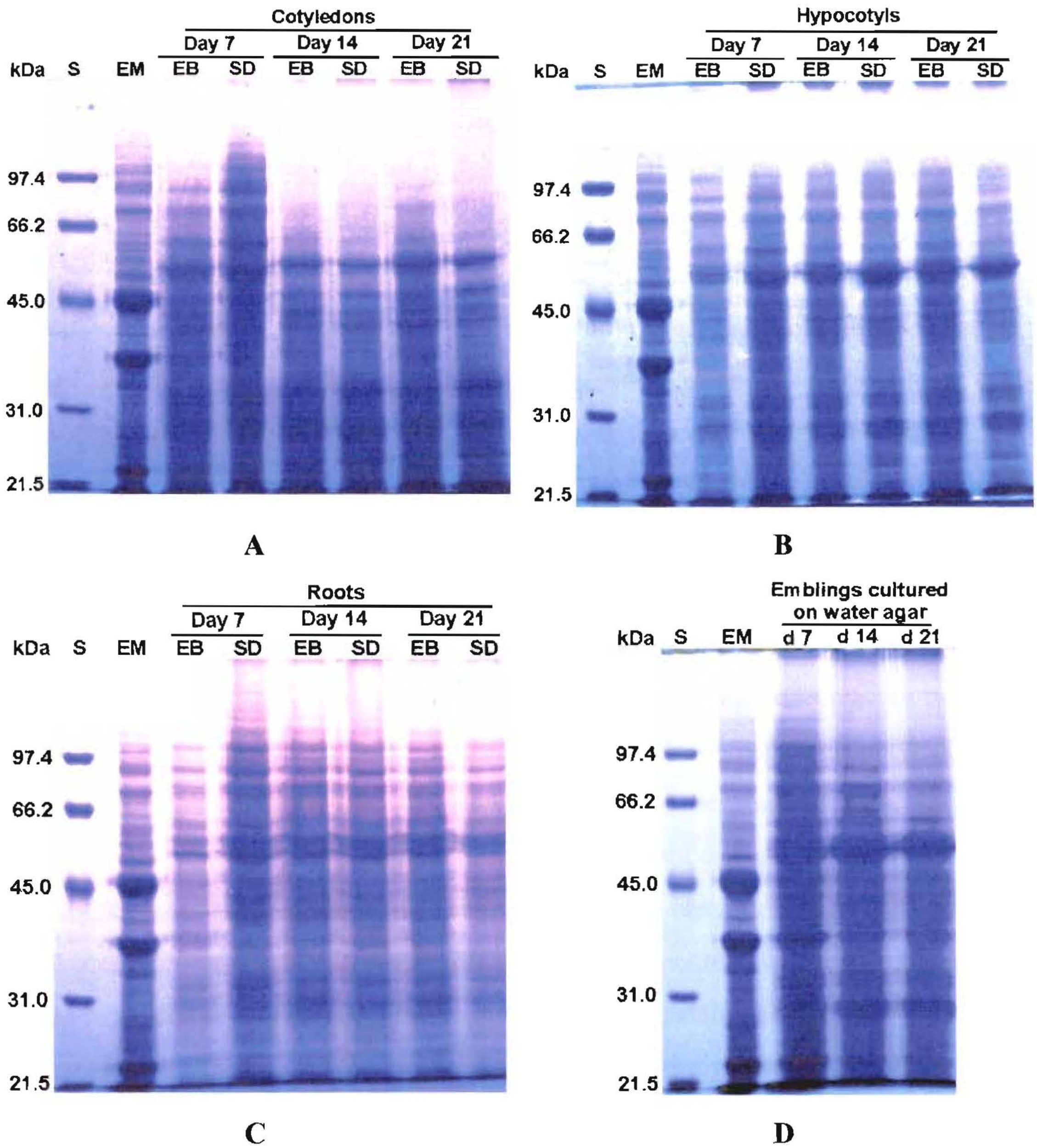


Plate 8. Coomassie blue staining after SDS-PAGE showing changes in proteins extracted from cotyledons (A), hypocotyls (B) and roots (C) of emblings grown on the LPSH2 medium and seedlings, as well as whole emblings grown on water agar (D) at day 0, 7, 14 and 21. Numerical values adjacent to the gels are molecular masses in kiloDaltons. S, protein standard; EM, isolated embryos before being cultured (day 0); EB, emblings; SD, seedlings of *Pinus radiata*

3.4.2 Time Courses of Soluble Sugar and Starch Changes

3.4.2.1 Soluble Sugar Assay

On fresh weight basis, at day 7 the soluble sugar concentration in the cotyledons or the hypocotyls of emblings was significantly lower (P<0.01) than the counterparts of the seedlings (Figure 27a and b). After this the soluble sugar concentration was significantly higher (P<0.01) in these parts of the emblings than in those of the seedlings. In the root, similar patterns of the soluble sugar concentration change were found in both emblings and seedlings. However, the concentrations of soluble sugar were significantly higher (P<0.01) in emblings than seedlings throughout the study period (Figure 27c).

The patterns of changes in soluble sugar concentration per seed part or embling /seedling are included for comparison (Figure 28a-d). In the cotyledons, a trend of increasing soluble sugar content was found in emblings while a decreasing trend was observed in seedlings. In general, the soluble sugar content per cotyledon was significantly lower (P<0.01) in emblings than seedlings throughout the study period. In the hypocotyl, the soluble sugar content per hypocotyl appeared increasing in emblings while in seedlings it rose during the second week before falling during the third week. In the root, an increase of soluble sugar content was found in emblings while in seedlings it fell during the second week before rising slightly during the third week. In general, the soluble sugar content per root was significantly higher (P<0.01) in emblings than seedlings throughout the study period. In the whole embling, a steady increase in soluble sugar content was observed, while in the seedling it increased sharply during the first week to a maximum at day 7 before decreasing gradually.

The soluble sugar content was significantly higher (P<0.01) when emblings were grown on the optimum medium (LPSH2) than on water agar medium (Figure 28d) throughout the study period.

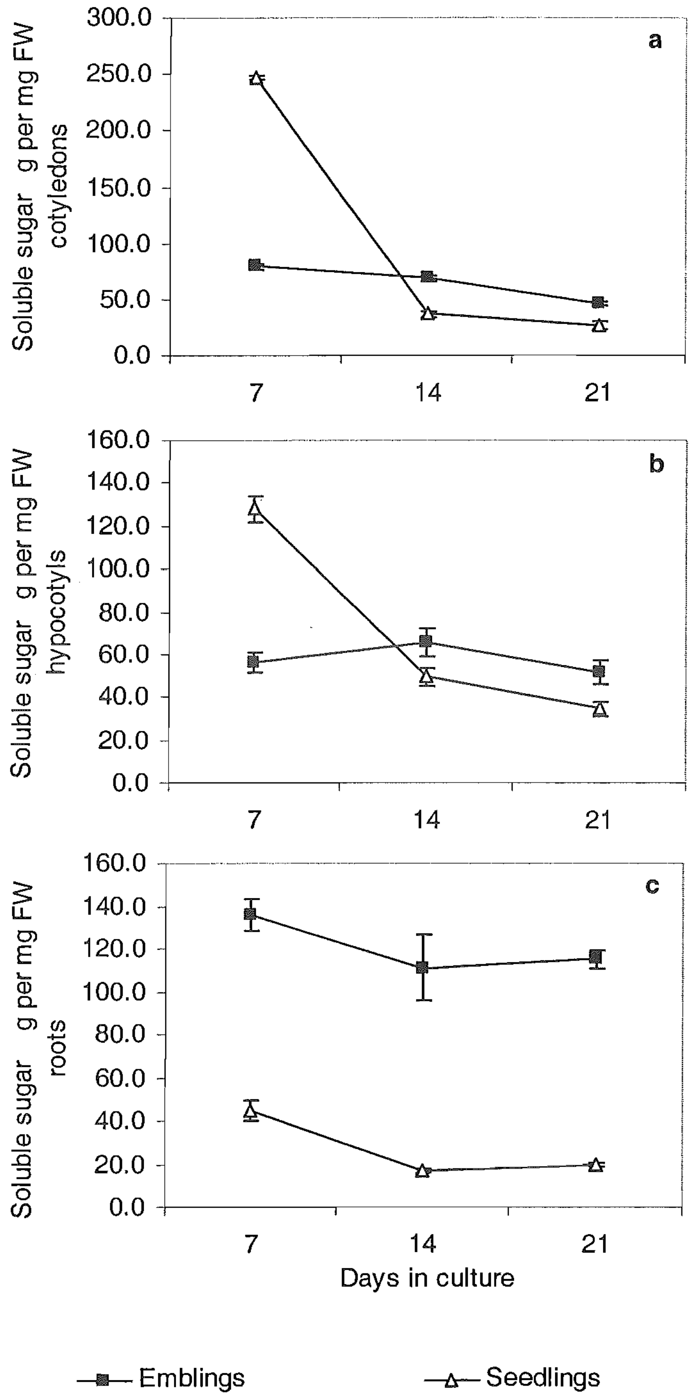


Figure 27. Time course of changes in mean soluble sugar concentrations, on fresh weight basis, in different seed parts of *Pinus radiata*. Error bars indicate the standard error of the mean and some are less than the size of a symbol.

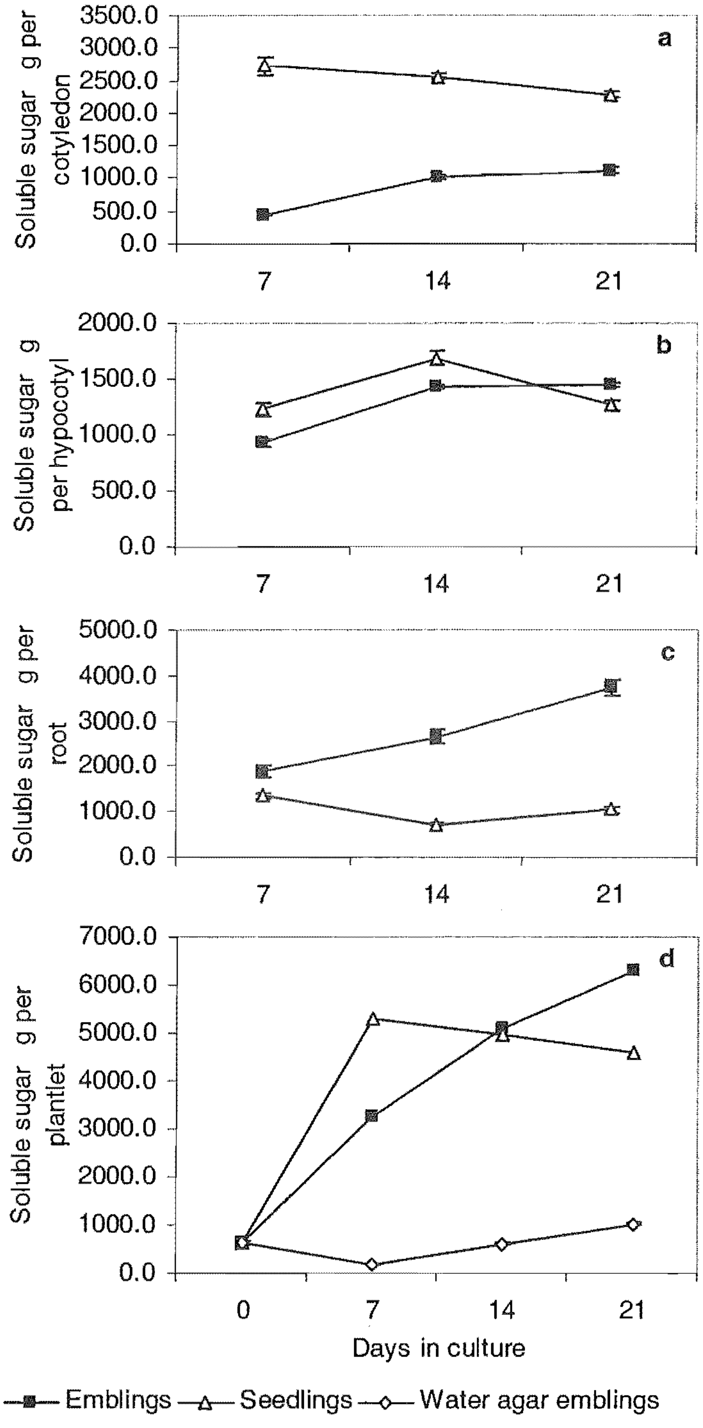


Figure 28. Time course of changes in mean soluble sugar concentrations per seed part or embling /seedling of *Pinus radiata*. Error bars indicate the standard error of the mean and some are less than the size of a symbol.

3.4.2.2 Starch Assay

On fresh weight basis, from day 7 to day 14 the amount of starch in the cotyledons was significantly lower ($P<0.01$) in emblings than in seedlings (Figure 29a). However, at day 21 the amount of starch was slightly higher ($P<0.05$) in emblings than in seedlings. In the hypocotyl, at day 7 the amount of starch was significantly lower ($P<0.01$) in emblings than in seedlings (Figure 29b). After this no big difference was found between emblings and seedlings. In the root, similar patterns of change in starch level were displayed by both emblings and seedlings (Figure 29c). However, the amount of starch were significantly higher ($P<0.01$) in the root of the emblings than in that of seedlings throughout the study period.

The patterns of changes in starch level per seed part or embling /seedling are also included for comparison (Figure 30a-d). In the cotyledons of the emblings, starch content was nearly unchanged throughout the study period while that in the cotyledons of the seedlings rose sharply during the second week to a maximum at day 14 before falling dramatically during the third week. In general, the starch content per cotyledon was significantly lower ($P<0.01$) in emblings than in seedlings in the first two weeks. In the hypocotyls of the emblings, the starch content per hypocotyl appeared to change little while that in the hypocotyls of seedlings fell sharply throughout the study period. In the roots of emblings, an increasing trend of starch content was found while in that of seedlings decreased slightly. In general, the starch content per root was significantly higher ($P<0.01$) in emblings than in seedlings throughout the study period.

The starch content was significantly higher ($P<0.01$) in emblings grown on the optimum medium (LPSH2) than on water agar medium (Figure 30d) throughout the study period. At day7 and day 14, starch content was higher in the seedlings than in the emblings. However, at day 21 while the emblings maintained the same level of starch as before, that in the seedlings decreased dramatically to a level lower than that of the emblings (Figure 30d).

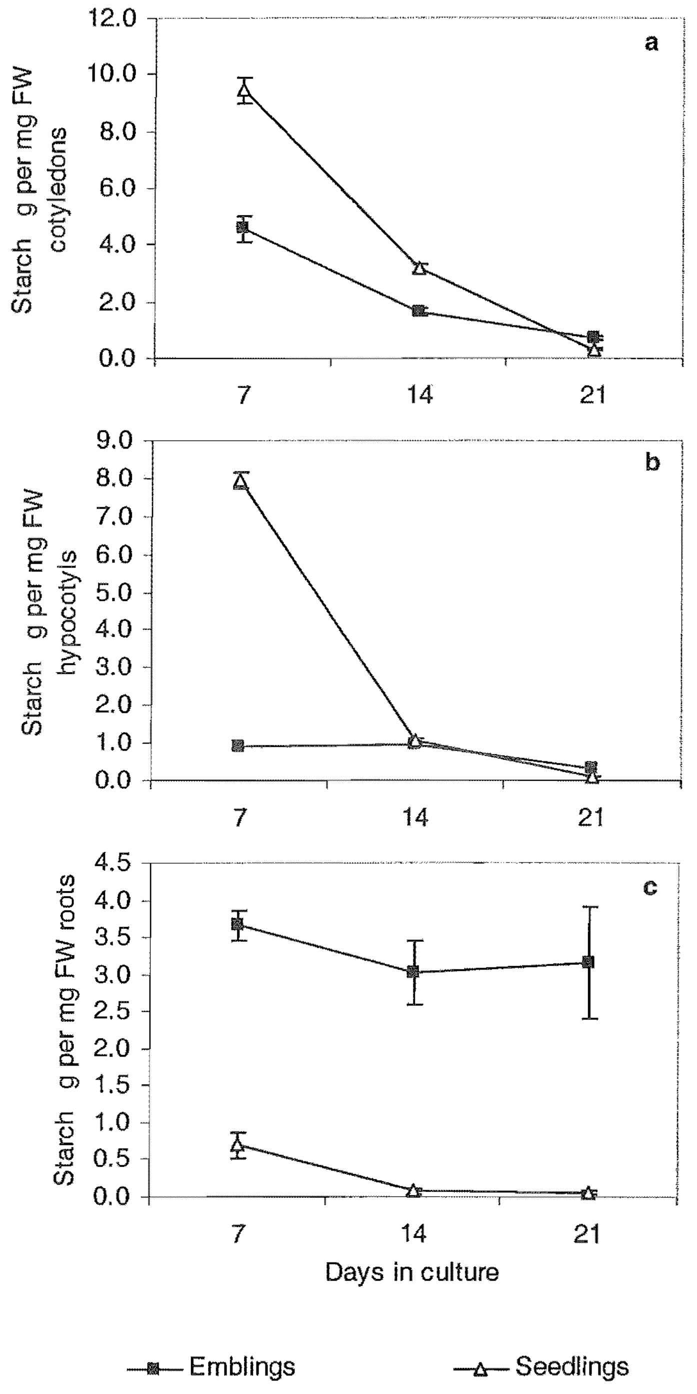


Figure 29. Time course of changes in mean amount of starch, on fresh weight basis, in different seed parts of *Pinus radiata*. Error bars indicate the standard error of the mean and some are less than the size of a symbol.

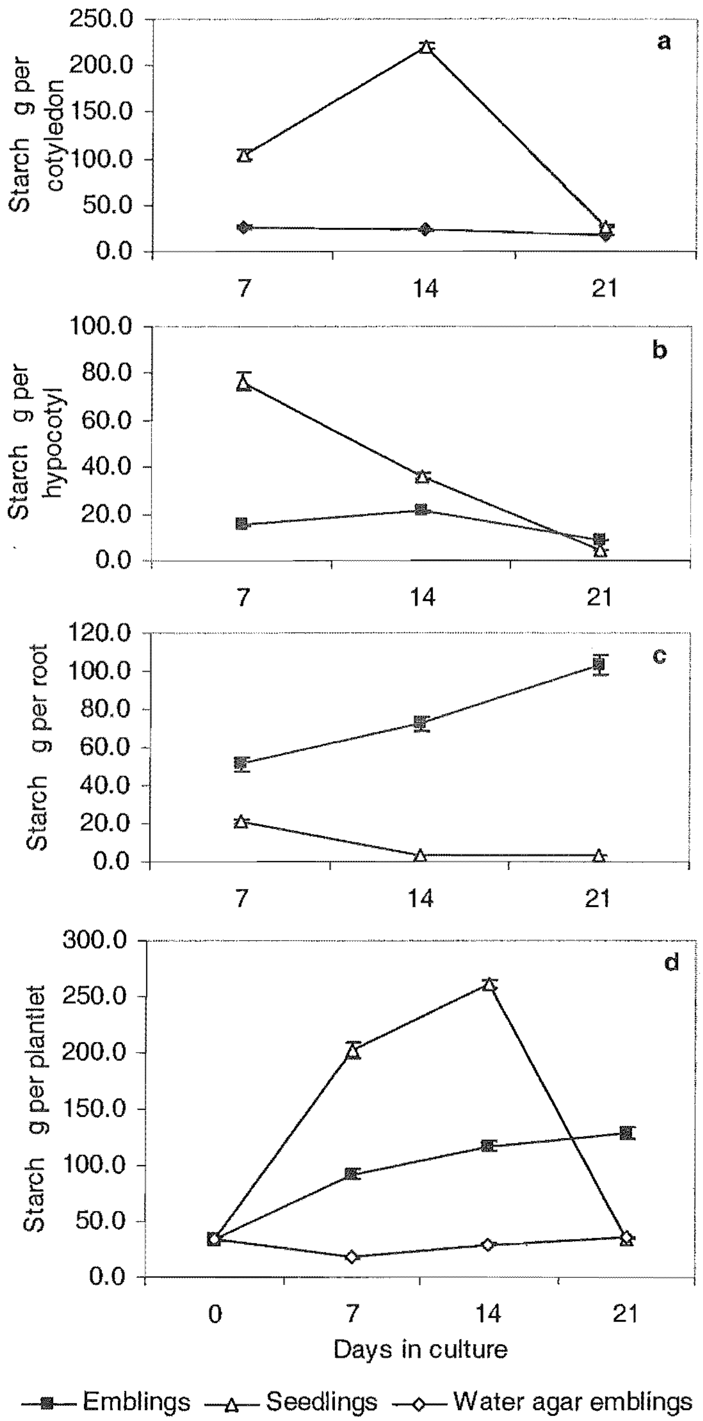


Figure 30. Time course of changes in mean starch concentrations per seed part or embling /seedling of *Pinus radiata*. Error bars indicate the standard error of the mean and some are less than the size of a symbol

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 Effect of Nutritional Factors on the Conversion of Isolated Embryos of *P. radiata*

The conversion of somatic embryos or isolated zygotic embryos of *P. radiata* into plantlets /seedlings takes place in the absence of the megagametophyte, which has storage reserves mainly in the form of lipids and proteins within the natural seeds. Nutritional factors therefore are critical to the conversion of isolated zygotic embryos into plantlets of *P. radiata*. It is not surprisingly that till the end of the experiment period no root developed from the isolated embryos cultured on the medium without external supply of nutrients, i.e. 0.8% (w/v) water agar medium (Plate 7), and the resultant plantlets were significantly smaller than those cultured on the optimum nutrient medium (LPSH2) (Figure 23d and 24d).

The optimum concentration of each nutrient for achieving maximum growth rates of isolated zygotic embryos of radiata pine varies considerably. In this study, the medium strength was firstly tested because it was the basis on which all other factors were applied. Modified Quoirin and Le Poivre (LP) medium (von Arnold and Eriksson 1981) and Schenk and Hildebrandt (1972) (SH) medium have both been used as a general tissue culture, micropropagation, or embryogenesis medium for *P. radiata* [Horgan (née Reilly) and Aitken 1981, Teasdale and Buxton 1986, Aitken-Christie *et al.* 1988, Gleed *et al.* 1995, Chandler and Young 1995]. However, preliminary experiments of this study (data not shown) indicated that the medium of modified LP medium salts (von Arnold and Eriksson 1981) combined with SH medium vitamins (Schenk and Hildebrandt 1972) was superior to the modified LP medium (von Arnold and Eriksson 1981) or SH medium (Schenk and Hildebrandt 1972) for the development of isolated radiata pine embryos. This study further revealed that the optimum medium strength for germination and growth rates of isolated mature radiata pine zygotic embryos was half strength of this combined medium (Figure 1, Table 1). Double strength (2x) medium did not gel well and delayed embryo germination, probably due to too high osmotic stress.

A suitable source of carbon energy is generally required for the cultivation of excised mature and immature zygotic embryos (Bhojwani and Razdan 1983). Sucrose, glucose, fructose, and maltose are the carbohydrates most commonly associated with development, maturation, germination, and conversion of embryos. Therefore, the addition of these nutrients to a medium may increase the capacity of embryo germination and early seedling growth. This thesis research demonstrated that no isolated mature embryo of *P. radiata* could germinate and grow normally on the medium without carbohydrate (Figures 2-5, Tables 2-5). Sucrose is by far the best form of carbohydrates and has been most commonly used for embryo culture in many Crops (Bhojwani and Razdan 1983, Lippmann. and Lippmann 1993, Nieves *et al.* 1998). Sucrose has two main roles when used under *in vitro* conditions; that is, as a carbon source and an osmotic agent (Kitto and Janick 1985). For this latter role the optimum concentration of sucrose varies with the stage of the embryo development. Mature embryos grow fairly well with 2% sucrose but younger embryos require higher levels of the carbohydrate (Bhojwani and Razdan 1983). Nieves *et al.* (1998) reported that 5% sucrose was adequate to induce good germination and conversion for naked *Cleopatra* tangerine zygotic embryos, and this concentration did not delay germination. Data presented in this thesis revealed that sucrose as well as glucose or fructose could serve as good carbon sources for the conversion of isolated zygotic embryos into plantlets of *P. radiata* (Figures 2-4, Tables 2-4), and the optimum concentration was 3% for sucrose, 2% for glucose, and 4% for fructose, respectively. Maltose was much inferior to sucrose, glucose or fructose as a carbon source and did not support isolated embryo growth well (Figure 5, Table 5). Sucrose is generally considered to be the carbohydrate that is transported to the seedling during the germination of oilseeds (Bewley and Black 1985), such as castor bean (Kriedemann and Beevers 1967). It has long been speculated that the end product of gluconeogenesis in the germinated conifer megagametophyte is also sucrose (Ching 1972, Kao 1973, Murphy and Hammer 1994, Stone and Gifford 1999). Kao and Rowan (1970) detected appreciable amount of glucose and fructose only in germinated seed but sucrose was found at all stages. They further found that sucrose increased twofold in the megagametophyte during stratification but decreased after germination when invertase activity appeared and growth began. Sucrose concentration increased in the embryo after stratification but remained high after germination. The relatively large amount of sucrose present in the megagametophyte during early seedling growth was also evident in loblolly pine (*Pinus taeda* L.) seeds (Stone and Gifford, 1999). The best *in vitro* growth of isolated embryos of *P. radiata* on sucrose-containing agrees well with these observations. This study also demonstrated that excessive concentration of sucrose, glucose or fructose depressed the germination and growth of isolated embryos, which imply an osmotic stress of high concentration of sugars. In addition, different kind of sugars as well as their concentrations seem to have different effect on various parts (i.e. cotyledon, hypocotyl and root) of emblings (Tables 2-4).

Common sources of organic nitrogen in nutrient media include amino acids, glutamine, asparagine and adenine. A source of organic nitrogen should not be necessary, but it is often beneficial to include a protein digest or L-glutamine (Gamborg and Shyluk 1981). The addition of amino acids, singly or in combination, to the culture medium may stimulate embryo growth. Generally, glutamine has proved to be the most effective amino acid for the growth of excised embryos (Bhojwani and Razdan 1983, Lippmann. and Lippmann 1993). Nieves *et al.* (1998) found that amino acid supplements (proline, glutamic acid and arginine) accelerated the conversion of naked *Cleopatra* tangerine zygotic embryos. Casein hydrolysate, an amino-acid complex, has been widely used as an additive to the embryo culture media. It was suggested that the promotive effect of casein hydrolysate might be due to some individual amino acids as well as their synergistic interactions (Sanders and Burkholder 1948). Teasdale and Buxton (1986) reported that addition of arginine monohydrochloride to the medium was beneficial for pine embryo development. The involvement of polyamines such as putrescine, spermidine, and spermine in the growth and development in woody plants have been well reviewed (Feirer 1995, Minocha *et al.* 1995). Additions of polyamines to the culture media affect the *in vitro* growth of woody plants. Many researchers indicate that polyamines may play many roles other than in the nitrogen nutrition of plant cells (Simola and Honkanen 1983, Slocum *et al.* 1984, Minocha *et al.* 1995). Data presented in this thesis indicated that, in general, there were a few or no significant benefits with the addition of organic nitrogen sources tested on the development of isolated zygotic embryos into plantlets of *P. radiata* (Figures 6-10, Tables 6-10). Casein enzymatic hydrolysate was found to be the best organic nitrogen source for isolated embryo culture of *P. radiata.* The addition of casein enzymatic hydrolysate not only supported embryo germination and early seedling growth but also promoted embryo conversion. However, the addition of arginine monohydrochloride had negative influence on the isolated embryo germination and early seedling growth of *P. radiata* (Figure 7, Table 7). Generally, during the germination and early seedling growth, the storage proteins are degraded to free amino acids, which are used in growth processes in the embryonic axes (Misra 1994, Stone and Gifford 1997). Arginine has been found to be a major component of seed storage proteins in maritime pine (*Pinus pinaster* Ait.) (Allona *et al.* 1992, 1994), Douglas fir *(Pseudotsuga menziesii* [Mirb.] Franco) (Leal and Misra 1993), and loblolly pine (*Pinus taeda* L.) (King and Gifford 1997), as well as of the soluble amino acid pools in germinated seeds of jack pine (*Pinus banksiana* Lamb.) (Ramaiah *et al.* 1971), stone pine (*Pinus pinea* L.) (Guitton 1964), and loblolly pine (*Pinus taeda* L.) (King and Gifford 1997). The results of this thesis research could hardly be explained by these observations. It is assumed that some differences may exist in the component of seed storage proteins between *P. radiata* and other coniferous species, and/or in the utilization of amino acids between isolated embryos and natural seed embryos during germination and early seedling growth. Therefore, comparative studies are needed on germination and early seedling growth kinetics between isolated embryos and natural seed embryos of different pine species to further evaluate a nutritive requirement for organic nitrogen in the culture medium.

4.2 Influence of Plant Growth Regulators on the Conversion of Isolated Embryos of *P. radiata*

Nearly all plant growth regulators tested were not very beneficial for the germination and early embling growth of isolated mature zygotic embryos of *P. radiata*, and some of them had negative effect (Figures 11-16, Tables 11-16). Adequate concentrations of GA_3 stimulate the germination of embryos a little bit earlier in comparison with the control (with no GA_3). However, BA, kinetin, IBA, and NAA obviously inhibited the germination and early embling growth. IAA at a range of concentration tested seemed to accelerate the germination process a little bit and stimulated the hypocotyl elongation but inhibited root elongation. Higher concentrations of NAA could cause isolated embryos to grow abnormally. In general, cotyledon and root tissues were more sensitive to plant growth regulators compared with the hypocotyl tissue. All these results indicate that exogenous growth regulators are not necessary for the normal development of isolated mature zygotic embryos of *P. radiata*. In fact, cytokinins and auxins are generally inhibitory for embryo germination and early seedling growth, especially cotyledon and root growth, of *P. radiata*.

The inhibition of root growth by low concentrations of auxin is a well-known phenomenon (Went and Thimann 1937). Slankis (1951) observed that concentrations of NAA and IAA above 0.5 mg/L commonly inhibited the growth of excised long roots of pine in liquid culture, while concentrations below 0.05 mg/L promoted elongation. Sacher (1956) also demonstrated the inhibitory effects of NAA (0.5 mg/L) on root growth of pine embryos in nutrient agar. This concentration caused the formation of protuberances on the primary roots, which Sacher interpreted as short roots. Brown and Gifford (1958) observed that with sucrose supplied through the cotyledons, the inhibitory effect of IAA on root growth was somewhat modified. There was an initial stimulation of root growth by the addition of gibberellic acid, but the effect was not sustained. The direct effect of the growth regulators tested on soybean embryo growth at the cotyledon stage mainly seemed to be an inhibitory one which became obvious at concentrations beyond the narrow stimulating range (Lippmann. and Lippmann 1993). Mattis *et al.* (1995) reported that gibberellins (GA) had little effect on excised embryo germination of Ilex in August through October. However, embryo germination of Ilex was completely inhibited by 3 μM GA in November and December. Llano-Agudelo *et al.* (1995) found that mature avocado embryo germination was 100% for embryos cultured with 0.3 mg IBA and 0.1-0.3 mg kinetin/L, IBA at concentrations >0.3 mg/L reduced germination. The addition of ABA (1 μM) to the culture medium delayed germination and conversion of naked *Cleopatra* tangerine zygotic embryos (Nieves *et al.* 1998). In most conifers, on the proliferation medium explant grows fast and produces only early stage embryos. Maturation of these embryos is similar to the maturation of zygotic embryos. Maturation medium for conifers commonly contains ABA and mature embryos must be transferred to a fresh medium lacking ABA and other plant growth regulators in order to initiate germination (Tautorus *et al.* 1991, Jain *et al.* 1995).

Although quantitative data are not available, it is assumed that in most cases embryos are autonomous for most of the plant growth regulators (Monnier 1978). This has been clearly shown at least for GA_3 in *Phaseolus*, where the suspensor fulfils the requirement of excised embryos for this hormone (Alpi *et al.* 1975). Apart from breaking dormancy in some plants (Buchheim *et al.* 1994), exogenous plant growth regulators are not necessary for the normal development of embryos, including isolated immature /mature zygotic embryos and mature somatic embryos. In fact, exogenous plant growth regulators are generally inhibitory for embryo growth and germination, and could sometime bring about structural abnormalities (Monnier 1978).

Studies have shown that ethylene may be required to promote callus growth /morphogenesis /somatic embryogenesis (Kumar *et al.* 1987, Auboiron *et al.* 1990, Kvaalen 1994) but it can also inhibit plant regeneration (Sethi *et al.* 1990, Kong and

Yeung 1994). The addition of $AgNO_3$, an ethylene biosynthesis /action inhibitor, to the culture medium may stimulate somatic embryo maturation and have no negative effects on embryo germination and conversion (Auboiron *et al.* 1990, Kong and Yeung 1994, Kong and Yeung 1995, Kim *et al.* 1997). Results from this study revealed that the addition of $AgNO_3$ at the concentrations tested to the culture medium caused negative effect on embryo germination and early seedling growth (Figure 17, Table 17). This indicates that the germination and growth of isolated embryos are not affected by ethylene and $AgNO_3$ is a harmful chemical to isolated embryo of *P. radiata.*

4.3 Effect of Physical Factors on the Conversion of Isolated Embryos of *P. radiata*

It is recognised that only mineral nutrients but not carbohydrates are normally supplied via the roots. This possibly explained that submerging the cotyledons of the embryo into the agar-gelled medium or embryos cultured in liquid medium showed better performance in comparison with the other treatment (Tables 18 and 19). Brown and Gifford (1958) found with *Pinus lambertiana* Dougl. embryos that nutrients, especially a carbohydrate, when supplied through the cotyledons exerted a much greater beneficial effect on both rate and duration of root growth than when they were supplied to the embryo directly via the root. Teasdale and Buxton (1986) similarly demonstrated with *Pinus radiata* D. Don embryos that artificial seeds (the embryos were inserted with cotyledons down into the agar-gelled medium in the artificial-seed capsules) showed better growth than the agar-planted embryos (the embryos were planted with radicle end down into the agar-gelled medium). Under *in vivo* conditions, the metabolite is, of course, supplied through the cotyledons by the megagametophyte for the germination and early seedling growth.

Although submerging the cotyledons of the embryo into the agar-gelled medium showed better root growth, the cotyledons did not grow as well as those of control (Plate 2) or those of the natural seed. Because most cotyledons failed to grow out off the medium, they did not spread out normally, and some had a purple pigment problem due to stress. The technique may need to be improved because Teasdale and Buxton (1986) reported 'artificial-seed'-derived plantlets were able to support and lift the capsule in the same way as does the natural seed coat. Embryos cultured in liquid medium grew better, but the germination rate was unacceptably low (55.6% by day 21) (Figure 19). Most of the ungerminated embryos exhibited aberrant growth, only formed swollen, stunted cotyledons and a hypocotyl, but no root growth (i.e. a stumpy root). These responses were frequently observed in cultures of somatic embryos (Egertsdotter 1999). Teasdale and Buxton also reported a similar observation to this study early in 1986, but they failed to obtain any normal plnatlets. Liquid medium with the polyurethane sponge support could improve the germination rate but the embryo growth was not comparable to the liquid medium only (Figure 19, Table 19). This suggests that embryos cannot take up nutrients efficiently through the sponge, which may retain some nutrients and affect their availabilities. The addition of suitable PEG (polyethylene glycol) 6,000 to the liquid medium seemed to ease the aberrant germination of isolated embryos and have no negative effect on embryo growth (Figure 20, Table 20). This may reveal some relationship between aberrant growth of isolated embryos and osmotic potential. Changes in osmolality affect, for example, not only Na^+, K^+-ATPase (one of the principal enzyme systems for active cation transport), and other important enzymes such as alkaline phosphatase but also RNA synthesis and polymerization in cells (Waymouth 1973). Liquid media will be ideal for automation in multiplying conifers via somatic embryos. One point for attention was that we should always keep part of cotyledons above the surface of the liquid medium during culture. Otherwise the plantlet would become purple and died probably due to the problem of aeration (Plate 4). Reducing the depth of liquid medium could more easily keep part of cotyledons above the surface of the liquid medium and hence avoid plantlet dying at a later culture period (Plate 3). It would be a good technique if we could find a special material, which can keep part of cotyledons always above the surface of the liquid medium and have no negative effect on nutrients uptake by embryos /seedlings during the culture period. Agitation can facilitate gaseous exchange of liquid medium, but it would not suit embryo culture because polarization of the tissue in relation to gravity is eliminated, and nutrient and growth regulator gradients are removed (Biondi and Thorpe 1981).

Light is generally not essential for growing callus or cell cultures. These cultures may grow equally well in the dark and light. However, for growth of embryo culture light is generally essential. Light has been shown to inhibit the germination of somatic embryos in most cases (von Arnold and Hakman 1988*b*) and it has been suggested that firstly somatic embryos are cultured in darkness until roots have started to elongate before they are transferred to light conditions. Results from this study show that the germination of isolated embryos of *P. radiata* was not affected by light condition, whereas light did influence embryo growth in different ways (Figure 21, Table 21). For root growth, 16-hour photoperiod appeared to be optimal, but for cotyledon development continuous light condition seemed to be better. In continuous darkness, the hypocotyl appeared to elongate more, but the cotyledon and root did not grow well. This indicates that light condition is important for the integrated growth of isolated embryos of *P. radiata*. Under *in vivo* conditions, the cotyledons not only take up the nutrients from the megagametophyte but also accumulate nutrients through photosynthesis once when they emerge out from the megagametophyte.

4.4 Biochemical Changes Associated With the Conversion of Isolated Embryos of *P. radiata*

Numerous investigations have been made on the biochemical changes associated with the germination and early seedling growth of natural conifer seeds. The primary storage reserves of many conifer seeds are lipids and proteins that are contained in lipid bodies and protein vacuoles (protein bodies), respectively (Gori 1979, Krasowski and Owens 1993). Although these reserves are present in both the embryo and the megagametophyte, most are stored in the megagametophyte (Ching 1966, Sasaki and Kozlowski 1969, Gifford 1988, Kovac and Kregar 1989, Stone and Gifford 1997). Several researchers found that later in the mature conifer seeds, lipid bodies, proteins, and starch were uniformly distributed in the megagametophyte. At maturity, starch was abundant in some regions of the embryo but not abundant in the megagametophyte (Hakman 1993, Krasowski and Owens 1993, Misra 1995). During germination and early seedling growth, storage lipids are converted to soluble carbohydrates (Ching 1966, Kao 1973, Stone and Gifford 1999), and storage protein reserves are degraded to amino acids (Durzan and Chalupa 1968, Salmia 1981, Lammer and Gifford 1989, King and Gifford 1997, Stone and Gifford 1997). The soluble carbohydrates (Firenzuoli *et al.* 1968, Murphy and Hammer 1988, Stone and Gifford 1999) and amino acids (Lammer and Gifford 1989, King and Gifford 1997, Stone and Gifford 1999) are transported to the seedling. In the seedling, they provide the building blocks and energy necessary for growth and the development of photosynthetic autonomy (Sasaki and Kozlowski 1969, Durzan *et al.* 1971, Murphy and Hammer 1994). In pinyon pine (*Pinus edulis* Engelm.) much of the exported sucrose is converted to starch, which accumulates mainly within the cotyledon and hypocotyl of the seedling (Murphy and Hammer 1994).

In contrast, there is little available information in the literature relating biochemical changes associated with the conversion of isolated embryos of *P. radiata.* Seed germination test from this study showed that the mechanical strength of the hard seedcoat strongly restricts the germination of *P. radiata* seeds (Figure 22). To make the results comparable, therefore, the hard seedcoat of natural seeds was removed in the biochemical change investigation experiment. Comparison studies on biochemical changes during germination and early embling /seedling growth showed that the patterns of total protein, soluble sugar, and starch content change were generally different between isolated embryos and natural seeds (Figures 26, 28 and 30). These imply that the ability of mobilization and utilization of available nutrients, especially carbohydrates, are different between emblings and seedlings. Thus emblings were smaller and lighter than seedlings (Figures 23 and 24). Total protein concentrations on the fresh weight basis (Figure 25) and their SDS-PAGE profiles (Plate 8) showed that there was little difference between emblings and seedlings. These possibly indicate that quantitative and qualitative changes and characteristics of proteins are similar between emblings and seedlings in particular sections. This further indicates that isolated embryos of *P. radiata* grew normally under *in vitro* condition. Although little difference was found on total protein composition and synthesis between emblings and seedlings, the patterns of free amino acids changes may be different between them. Therefore, a future investigation on the difference in changes of free amino acids during germination and early embling /seedling growth between isolated embryos and natural seeds may be meaningful. The different patterns in the changes of soluble sugar (Figure 27) and starch (Figure 29) on the fresh weight basis with time between emblings and seedlings may be due to the different sources of nutrients and their availabilities. In natural seeds, nutrients are supplied through the cotyledons by the megagametophyte, in which the levels of available nutrients increase sharply with radicle emergence but then decrease steadily as it becomes in contact only with the cotyledons (Pitel and Cheliak 1988, King and Gifford 1997, Stone and Gifford 1997, Stone and Gifford 1999). Conversely, it is possible that for isolated embryos nutrients are likely to be in excess in the medium and taken up steadily mainly through the root during the culture period. These possibly accounted for significantly higher soluble sugar /starch concentration on fresh weight basis in seedlings at day 7 than at day 14 /day 21. These may also explain that at day 7 in cotyledon /hypocotyl section soluble sugar /starch concentration on fresh weight basis was significantly higher in seedlings than in emblings, whereas in root section it was significantly lower in seedlings than in emblings. Future studies on mobilization, utilization and accumulation of carbohydrates, as well as relevant enzymes activity in embings, particularly those in the roots under *in vitro* condition, are needed.

4.5 About Endogenous Contaminations

Because *P. radiata* is a perennial plant, it is generally thought to be more difficult to obtain axenic cultures than other annual plants. In series experiments, a common interesting phenomenon was observed, in that when isolated embryos failed to grow early in culture, severe microbial contamination became evident. Conversely, when isolated embryos cultured on the media that were suitable for isolated embryo germination and growth, contamination was seldom a problem. This suggests that there is a growth competition between isolated embryos and its endogenous microorganisms. In an attempt to solve this problem, 0.1% PPM (plant preservative mixture) (Austratec Pty Ltd., Australia) had been applied, but it did not have any effect (data not shown).

4.6 Conclusions

1. Of the all factors tested, nutritional factors were the most important to the germination and early embling growth of isolated embryos of *P. radiata* under *in vitro* condition.

2. The optimum medium strength for the germination and growth of isolated embryos of *P. radiata* was a half strength of the medium consisting of modified Quoirin and Le Poivre (LP) salts (von Arnold and Eriksson 1981) and Schenk and Hildebrandt (1972) (SH) vitamins.

3. Sucrose as well as glucose or fructose could serve as carbon sources for the germination and early seedling growth of isolated embryos of *P. radiata*. The optimum concentration was 3% for sucrose, 2-3% for glucose and 2-5% for fructose.

4. In general, there were a few or no significant benefits with the addition of organic nitrogen sources tested on the performance of isolated zygotic embryos into plantlets of *P. radiata*. Casein enzymatic hydrolysate was found to be the best organic nitrogen source for isolated embryo culture of *P. radiata*.

5. Nearly all plant growth regulators tested were not beneficial for the germination and early seedling growth of isolated mature zygotic embryos of *P. radiata*, and some of them had negative effects. Only GA_3 seemed to stimulate embryos to germinate a little bit earlier in comparison with the control.

6. Submerging the cotyledons of the isolated embryo into the agar-gelled medium showed better growth in comparison with the control.

7. Isolated embryos cultured in liquid medium grew better, but the germination rate was unacceptably low. The addition of PEG (polyethylene glycol) 6,000 to the liquid medium could increase the germination rate and had no negative effect on embryos.

8. Light conditions did not affect the germination of isolated embryos of *P. radiata*, but did influence their growth. 16-hour or more photoperiod appeared to be good for the integrated growth of isolated embryos; but continuous darkness was not ideal for the embling establishment from the isolated embryo.

9. The mechanical strength of the hard seedcoat strongly restricted the germination of *P. radiata* seeds.

10. Isolated embryos cultured on the optimum medium (LPSH2) grew well and normal, but smaller than natural seedlings.

11. Studies on biochemical changes during germination and early embling or seedling growth showed that the patterns in changes of total protein, soluble sugar, and starch content were generally different between emblings and seedlings. However, on fresh weight basis, total protein concentrations and their SDS-PAGE profiles showed that there was little difference between emblings and seedlings.

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Appendix 1

1. Modified Quoirin and Le Poivre (von Arnold and Eriksson 1981) (LP) Medium Salts Stock Solutions

<u>LP Major Salts Stock Solution</u>

	g/L
KNO_3	36
$Ca(NO_3)_2 \cdot 4H_2O$	24
NH_4NO_3	8
$MgSO_4 \cdot 7H_2O$	7.2
KH_2PO_4	5.4

Stored at 4 °C.

<u>LP Minor Salts Stock Solution</u>

	g/L
$ZnSO_4 \cdot 7H_2O$	8.6
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	20.0
$CuSO_4 \cdot 5H_2O$	0.25
KI	0.08
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CoCl_2 \cdot 6H_2O$	0.025

Stored in 50 mL aliquots in the freezer. When required, take one 50 mL aliquot and make up to 500 mL with distilled water (dH_2O). Stored at 4 °C.

<u>LP Iron Stock Solution</u>

	g/L
$FeSO_4 \cdot 7H_2O$	1.5
Na_2EDTA	2

Boil EDTA with dH_2O, cool and add $FeSO_4 \cdot 7H_2O$. Stored in a brown bottle at 4 °C.

2. Schenk and Hildebrandt (1972) (SH) Medium Vitamins Stock Solution

	mg/L
Thiamine HCl	500
Nicotinic acid	500
Pyridoxine HCl	50

Stored at 4 °C.

3. LPSH1 Medium

LP Major Salts Stock Solution	50 mL
LP Minor Salts Stock Solution	10 mL
LP Iron Stock Solution	20 mL
SH Vitamins Stock Solution	10 mL
Inositol	1 g
Sucrose	30 g
L-glutamine	750 mg (5.13mM)
Casein enzymatic hydrolysate	250 mg
GA_3	0.10 mg (0.29 μM)
Agar	8 g
dH_2O	make up to 1.0 litre

pH of the medium is adjusted to 5.6-5.8, then the medium is autoclaved.

4. LPSH2 Medium

LP Major Stock Solution	25 mL
LP Minor Stock Solution	5 mL
LP Iron Stock Solution	10 mL
SH Vitamins Stock Solution	5 mL
Inositol	0.5 g
Sucrose	30 g
Casein enzymatic hydrolysate	250 mg
GA_3	0.20 mg (0.58 μM)
Agar	8 g
dH_2O	make up to 1.0 litre

pH of the medium is adjusted to 5.6-5.8, then the medium is autoclaved.

Appendix 2

1. Plant Growth Regulator Stock Solutions

GA$_3$ (gibberellic acid) stock solution: 1 mg/mL in dH_2O, initially dissolved in ethanol (EtOH). Stored at 4 °C.

BA (6-benzyl adenine) stock solution: 1 mg/mL in dH_2O, initially dissolved in 1 M NaOH. Stored at 4 °C.

Kinetin stock solution: 1 mg/mL in dH_2O, initially dissolved in 1 M NaOH. Stored at 4 °C.

IBA (indole-3-butyric acid) stock solution: 1 mg/mL in dH_2O, initially dissolved in EtOH or 1 M NaOH. Stored at 4 °C.

IAA (indole-3-acetic acid) stock solution: 1 mg/mL in dH_2O, initially dissolved in EtOH or 1 M NaOH. Stored at 4 °C.

NAA (α-naphthaleneacetic acid) stock solution: 1 mg/mL in dH_2O, initially dissolved in 1 M NaOH. Stored at 4 °C.

2. Starch Assay Iodine Stock Solution

Iodine	0.06 g
Potassium iodide (KI)	0.60 g
dH_2O	10 mL

0.60 g of KI was dissolved in 10 mL of dH_2O, then 0.06 g of iodine was dissolved in the KI solution. Stored in a brown bottle at 4 °C.

Appendix 3

1. Protein Assay Stock Solutions

1M Tris-HCl, pH 8.8

	per 250 mL
Trizma Base	30.285 g

Dissolved in dH_2O, pH adjusted to 8.8 with concentrated HCl. Stored at 4 °C.

1M Tris-HCl, pH 6.8

	per 100 mL
Trizma Base	12.114 g

Dissolved in dH_2O, pH adjusted to 6.8 with concentrated HCl. Stored at 4 °C.

30% Acrylamide: Bis Solution

	per 100 mL
acrylamide	29.2 g
bis	0.8 g

Dissolved in dH_2O, Stored in a brown bottle at 4 °C.

10% Sodium Dodecyl Sulfate (SDS) Solution

	per 100 mL
SDS	10 g

Dissolved in dH_2O, Stored at room temperature.

Coomassie Blue Reagent (Bradford 1976)

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 mL 95% ethanol, 100 mL 85% (w/v) phosphoric acid was added to this solution. This resulting solution was diluted to a final volume of 1 litre. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (v/v) ethanol and 8.5% (w/v) phosphoric acid. The solution was filtered and left to stand for 3 days before use.

2. Recipes for Laemmli Gels

10% Acrylamide Separating Gel

dH_2O	8.0 mL
1M Tris-HCl, pH 8.8	11.25 mL
30% acrylamide: bis	10.0 mL
10% SDS	300 μL
10% ammonium persulfate (0.1 g + 1.0 ml dH_2O)	300 μL
TEMED	15 μL
Total volume	30 mL

3% Acrylamide Stacking Gel

dH_2O	7.5 mL
1M Tris-HCl, pH 6.8	1.25 mL
30% acrylamide: bis	1.0 mLl
10% SDS	100 μL
10% ammonium persulfate (0.1 g + 1.0 ml dH_2O)	40 μL
TEMED	15 μL
Total volume	10 mL

Electrophoresis Buffer

	per 1000 mL
Trizma base	4.54 g
Glycine	21.6 g
SDS	1.5 g
dH_2O bring volume to	1000 mL

Stain (Coomassie Brilliant Blue)

	per 100 mL
Absolute methanol	50 mL
Glacial acetic acid	10 mL
Coomassie Brilliant Blue	0.1 g
dH_2O	40 mL

Destain (5% Methanol, 10% Acetic Acid)

	per 1000 mL
Methanol	50 mL
Glacial acetic acid	100 mL
dH_2O	850 mL

Sample Buffer

	per 20 mL
dH_2O	8.0 mL
1M Tris-HCl, pH 6.8	2.5 mL
Glycerol	4.0 mL
10% SDS	4.0 mL
2-B-mercaptoethanol	1.0 mL
0.1% Bromophenol Blue	0.5 mL

Aliquot (1 mL) into eppendorfs and stored at -20 °C.